THE USE OF CYCLOSPORIN A AND AZATHIOPRINE IN CLINICAL KIDNEY TRANSPLANTATION

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De klinische toepassing van cyclosporine A en van azathioprine na niertransplantatie

PROEFSCHRIFT

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ter afsluiting

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CHAPTER 1

INTRODUCTION

Transplantation of a kidney is now widely accepted as the treatment of choice for most patients with end-stage chronic renal failure.
The development of the surgical technique needed for transplanting kidneys began in the early 1900's. At the end of the first decade of this century allogeneic dog kidneys were already successfully transplanted due to reliable suture techniques for vascular anastomosis, but allograft survival was poor [1, 2]. In 1923, Williamson stated that the failure of renal transplants in animals was attributable to a biologic incompatibility between donor and recipient [3]. It was not until the late 1940's that Medawar and collegues demonstrated the immunological nature of the allograft rejection [4]. The fundamental basis of the immune system proved to be tolerance to self tissues and lack of tolerance to foreign antigens resulting in an appropriate immune response to allografts.
Although in humans the technical feasibility of renal transplantation was demonstrated from successful renal homograft transplantation between genetically identical (monozygotic) twins in the 1950's [5], the immunological barrier remained the major obstacle to the widespread use of transplantation as replacement therapy for terminal organ failure. To allow for a wider clinical application of renal transplantation, the immune response to alloantigens which causes donor graft rejection when donors and recipients are not genetically identical, had to be suppressed.

IMMUNOSUPPRESSION

Initial attempts to depress the immune system and to inhibit rejection employed total body irradiation in various doses, but provided only minimal success. It soon became apparent that with this technique, if a high enough dose of irradiation was given, it was possible to prevent graft rejection [6]. However, at the same time this led to the destruction of the immune system and the recipient of the graft usually died of infection. When lower doses of irradiation were used, immunological competence recovered and the graft was still rejected. This highlights the major problem in the management of non-specific immunosuppression: too much of it and infection supervenes, too little and rejection destroys the graft. This delicate balance always threatened the further developments of immunosuppression.

The recognition of drugs with immunosuppressive properties led to a major advancement in the field of organ transplantation. The principal goal was to find agents making the immune mechanism unresponsive to the specific alloantigenic stimulus of the engrafted organ, while sparing normal host resistance. In addition, pharmacological immunosuppression allowed daily modification of the amount of immunosuppression given, so that episodes of rejection or infection could be dealt with as and when they occurred.

CORTICOSTEROIDS

The first step toward this objective was the observation that the rejection of primary skin grafts was significantly delayed by steroid therapy [7, 8]. Even second-set rejections in pre-sensitized recipients could be favorably modified [9]. The ability of corticosteroids to prevent or suppress inflammation whether the provoking agent is infectious, immunological, mechanical, chemical or radiant, is known for a long period. The mechanisms involved in these actions and interactions are not yet fully discerned, but broadly they can be said to affect both leucocyte traffic and function [10]. Despite an increase in the number of freely circulating neutrophils produced by the bone marrow, corticosteroids decrease the response to chemotactic factors, thereby inhibiting the migration of neutrophils and macrophages to the site of tissue inflammation. In addition to steroid induced lymphocytopenia (largely due to redistribution of recirculating lymphocytes into bone marrow), corticosteroids have profound effects on the lymphocyte function. They inhibit the proliferative response of T lymphocytes to both mitogenic and antigenic stimuli. Also, macrophages are made less responsive to stimulating lymphokines and their production of Interleukine-1 is reduced [11]. The side effects of corticosteroids in renal transplant recipients, especially when using high doses, are well-known. A high incidence of avascular osteonecrosis was reported [12]. The increase of the recipient's susceptibility to bacterial infections is mostly due to the failure to export neutrophils into the tissues and to the reduced macrophage function. In addition, corticosteroids have important metabolic side effects (e.g. glucose intolerance, osteoporosis, hypertension).

AZATHIOPRINE

The second important development was the recognition of the immunosuppressive properties of 6-mercaptopurine (6-MP). During its early use in cancer therapy it was noted that many patients developed symptoms of immunosuppression [13]. In animal experiments 6-MP was shown to mitigate rejection of rodent skin grafts and kidney allografts in dogs [14, 15, 16]. Mercaptopurine is an anti-metabolite drug which can be classified as phase specific. It exerts its toxic effect at the cellular level during a select period of the cell-cycle. The four phases of the cell-cycle are G1

(pre-DNA synthesis), S (DNA-synthesis), G2 (post DNA-synthesis) and M (mitosis) [17]. Mercaptopurine acts as a S-phase toxin. Azathioprine (AZA), the nitro-imidiazole derivate of the purine antagonist 6-MP, has similar immunosuppressive effects but less toxicity [18]. Administered orally, AZA is rapidly converted to 6-MP which is subsequently converted into 6-thio-inosinic acid. The latter compound is the active form which functions as a competitive enzyme inhibitor to block the synthesis of inosinic acid, the precursor of adenylic acid and guanylic acid [19]. The major manifestation of this interference with DNA-synthesis is a decrease in the rate of cell division. The prime targets for the toxic effect are actively replicating cells.

Although the precise mode of action of AZA on the immune system is not completely known, it is thought that AZA primarily affects the rapidly dividing immature cells of the immune system and has only little impact on the mature components such as antigenspecific memory cells and end-stage cells such as plasma cells [20]. Recently, it appears that AZA may have selective effects on a limited number of components of the immune system, specifically inhibition of T suppressor cell function and natural cytotoxicity [21, 22].

The cytotoxic properties of AZA are not only related to drug concentration but also to the duration of cell exposure. It is known that a single massive dose of AZA results in little toxicity, while longterm administration frequently results in significant toxic effects [23].

The most frequently occurring side effects of AZA are bone marrow depression, bacterial and viral infection, gastro-intestinal intolerance and hepatotoxicity. Granulocytopenia is the most prominent manifestation of the bone marrow depression. Occasionally trombocytopenia and anemia can occur. The incidence and severity of the different types of therapeutic complications appear to be related to drug dose and drug combination, especially the combination with steroids. The number of leucocytes and especially of granulocytes is an important parameter for dose adjustment.

Bacterial and viral infections are a matter of concern in transplantation [24]. They are the major contributory factors in morbidity and mortality during the first three months after renal transplantation, probably as a direct result of induced granulocytopenia.

Another major concern of immunosuppressive therapy is the induction of malignancy [25]. In 5-6% of the renal transplant patients receiving longterm AZA a malignant tumor develops; in other words, there is a 100-fold increase compared with the general population [26]. Most tumors are of epithelial origin (skin, cervix). Also there is an increased occurrence of Epstein-Barr virus associated lymphoma's and other lymphoproliferative syndromes [27].

In the initial clinical attempts AZA, when used alone, was not effective in preventing allograft rejection [28]. A new era of transplantation was not entered until it was recognized that AZA and corticosteroids had an additive (or possibly synergistic) effect [29]. Dual therapy of AZA in combination with corticosteroids

was needed to circumvent episodes of rejection and to prolong renal graft survival [30]. Early clinical protocols used AZA immediately following transplantation and added high doses of prednisone with the first symptoms of rejection. Because the vast majority of renal allograft recipients underwent rejection episodes, the protocol was soon modified to begin treatment with both drugs at the time of the surgical procedure. A randomized prospective study comparing high and low dose prednisone after transplantation showed no difference in graft survival and renal transplant recipients were maintained on low levels of prednisone therapy [31, 32]. However, during acute graft rejection episodes large doses of prednisone (pulse therapy) were employed. This double drug therapy has provided the basic immunosuppressive drug regimen throughout the world for almost twenty years.

The results of treating renal failure by kidney transplantation improved steadily over the 1960's both in terms of graft survival and patient survival. These improvements were due to an increased experience in the management of the potentially lethal immunosuppressive drugs and the concurrent improvement in dialysis techniques for fall-back maintenance in the event of uncontrollable rejection. In the early 1970's longterm renal graft function was obtained in approximately 70% of living related donor transplants [33]. The overall success rate with cadaveric renal transplantation, however, was significantly less with close to 50% graft loss within the first year in most centers, rejection accounting for the vast majority of these losses [34, 35]. Because cadaveric grafts constituted the major proportion of kidney transplants, modifications to the combined AZA and prednisone protocol have been sought. Although patient mortality has decreased but the morbidity of chronic immunosuppression (particularly with high-dose steroid therapy) has been well recognized, even after successful transplantations. About 40 percent of the longterm graft recipients was invalidated by avascular osteonecrosis, particularly of the hip [12]. The recognized need for a better therapy prompted a number of deviations from the original immunosuppressive therapy.

IMMUNOGLOBULINS

Between 1963 and 1979 a number of modifications of, or additions to the original double drug regimen was introduced. A promising approach was the use of antilymphocyte serum or its derivatives (anti-lymphocyte globulin (ALG) and antithymocyte globulin (ATG)) to deplete circulating lymphocytes. ALG was given as a temporary supplement to AZA and prednisone during the first period posttransplantation decreasing the incidence of rejection [36]. However, after discontinuation of ALG there has been a moderately high rate of delayed rejection episodes and the one year graft survival after cadaveric renal transplantation did not improve with this approach [37, 38, 39]. The use of ALG and ATG was not without side effects of whom fever and chills were the most prominent next to serum sickness. Also an increased incidence of cytomegalovirus infections was noted during ALG in combination with AZA and prednisone therapy, necessi-

tating discontinuation of immunosuppressive therapy and risking graft loss [40]. Temporary lymphoid depletion by thoracic duct drainage (depletion of recirculating T lymphocytes) in the preparation of patients for cadaveric transplantation or total lymphoid irradiation before grafting has not been widely used in renal transplantation because of their inconvenience and the difficulty of reversing the effect in the event of a complication [41, 42, 43]. The value of splenectomy as an adjunct to drug therapy remained controversial [44, 45], while the risk of lethal bacterial infections after splenectomy was substantial [46]. More recently monoclonal antibodies which bind to specific determinants on T lymphocytes, were used in short courses in experimental therapy trials to treat primary rejection events [47] and also to prevent rejection in the immediate posttransplant period [48].

HLA-MATCHING AND BLOOD TRANSFUSIONS

Another approach to improve cadaveric graft survival was to provide a more advantageous biological environment for the grafts by exploiting the developments in tissue typing and matching or by systematically conditioning prospective renal allograft recipients with preoperative blood transfusions. Results of typing and matching were shown to be relative advantages to the longterm survival of the graft and the recipient [49]. The latter practice of conditioning by transfusion has caused an increased success rate in those patients not accidentally sensitized during their preparation for transplantation. Also in living related donor transplantation the preconditioning of recipients with AZA and donor specific blood transfusions (DST) has improved the rates of renal graft survival [50].
Despite considerable amounts of effort, time and money invested, the results in kidney transplantation had only marginally improved by the end of the 1970's. Just over half the kidneys transplanted maintained function for more than one year. However, almost one half of the kidney grafts were still being lost with rejection accounting for the vast majority of these losses. For the expansion of renal transplantation it was necessary to hope for better immunosuppressive drugs. Against this background the introduction of cyclosporin A on the immunosuppressive battlefield must be viewed.

CYCLOSPORIN A

In the early seventies the fungus Tolypocladium inflatum Gams was isolated from a soil sample obtained from the Hardanger Vidda, a large treeless highland plateau in the southern part of Norway. The most important metabolite was cyclosporin A (CsA). This compound was first isolated as part of a search for biologically produced antifungal agents. It proved to have only a mild antifungal activity but in an additional screening program it was discovered to have immunosuppressive

properties [51]. CsA is the representative of a new group of structurally related cyclic undecapeptides, obtained as fermentation products of Tolypocladium inflatum Gams (formerly known as Trichoderma polysporum Rifai) and Cylindrocarpum lucidum Booth. A hitherto unknown nine-carbon open-ring amino acid at position one is unique to the cyclosporins. This novel amino acid is necessary but not sufficient for activity. Especially the three-dimensional molecular structure determines the immunosuppressive action. The formula of CsA is C_{62} H_{111} N_{11} O_{12} and its molecular weight 1202 Da.

Pharmacokinetics

The hydrofobic nature of CsA renders it insoluble in aqueous solutions and CsA must therefore be dissolved in lipids or organic solvents before administration. After oral ingestion CsA is absorbed from the small intestine and enters the lymphocyte-rich environment of the thoracic duct where it may exert its first immunosuppressive effect on the lymphocyte population [52]. From the thoracic duct CsA is delivered into the systemic circulation. With prolonged use of the drug a progessive increase in bio-availability was demonstrated. This may reflect an improvement of gastro-intestinal dysfunction due to the pretransplant uremia or an induction of a CsA transport mechanism in the small intestine [53]. Approximately ninety percent of plasma CsA is protein bound, in large part to the lipoprotein fraction. Erythrocytes demonstrated a tenfold greater CsA binding affinity than T or B lymphocytes [54]. Chronic administration results in accumulation of tissue stores in fat and skin with high levels also seen in kidney, liver, pancreas and adrenals [55]. Saturation of the peripheral compartment or increase in absorption eventually results in decreased requirements with longterm use to maintain serum levels. CsA is metabolized in the liver by the cytochrome-P450 system and secreted by the liver via the bile into the feces. Enterohepatic recirculation has been reported [53]. Only a small proportion up to 10% of the total drug metabolites is excreted into the urine [54].

Because of the marked variability in gastro-intestinal absorption, hepatic biotransformation, excretion and tissue response, considerable efforts have been made to measure CsA levels in patient blood for monitoring adequacy of treatment and avoiding drug toxicity. Currently three techniques are in routine use, namely radioimmunoassay (RIA), fluorescence-polarization-immunoassay (FPIA) [56] and high performance liquid chromatography (HPLC). HPLC measures parent drug only whereas the polyclonal antiserum used in the RIA and FPIA detects both CsA and its metabolites resulting in trough levels which are two to four times higher. Whole blood levels generally are 3 to 5 times higher than plasma levels due to the high affinity of CsA for erythrocytes [57]. Recently, the polyclonal antiserum used in the RIA and FPIA has been replaced by monoclonal antibodies to CsA. No uniformly safe trough drug concentration range, which avoids toxicity while assuring immunosuppression, has been established [58]. Because of the wide inter- and intra-individual variation in CsA levels achieved with a given dose, drug

level monitoring by serial determinations of trough levels appears to afford the simplest, albeit imperfect, guide to treatment.
A number of drugs have been reported to interact with CsA. These interactions result in either an increase or a decrease in CsA concentrations. Some can be explained by an influence on hepatic microsomal cytochrome-P450 activity. Substances known to be inhibitors of this enzyme such as ketaconazole increase CsA levels while enzyme-inducers (rifampicine and diphenylhydantoin) decrease CsA levels [59, 60].

Immunological action

Initial animal experiments by Borel and co-workers showed a suppressive effect of CsA on antibody production in mice [51]. Its failure to suppress antibody synthesis to lipopolysaccharide antigens (a direct stimulation of B cells) in thymectomized mice suggested a selective effect on T cells [52]. Specific T cell-dependent responses, as delayed-type cutaneous hypersensivity to oxazolone and tuberculin, were effectively inhibited by CsA [61]. Time-course studies showed that although administration of CsA at the time of antigenic challenge suppressed antibody production, ongoing antibody responses were not impaired. Suppression only occurred when the drug was present at initiation or in the early stages of the immune response [62]. The effector function of previously activated cytolytic or suppressor T cells was not altered. Similarly, pretreatment of T lymphocytes followed by removal of the drug before antigen exposure was without an effect on subsequent responses [63]. These experiments showed that CsA prevented induction of T cell-dependent antigenic responses and that CsA had little or no effect on B lymphocyte responses. The T lymphocytes seemed to be the primary immunological target of CsA.
The model of T cell activation sequence requires expression and recognition of antigens and cell-derived growth factors by immunocompetent cells. Antigens are presented by so-called antigen presenting cells, having class II major histocompatibility antigens on their surface (macrophages, dendritic cells and Langerhans cells), to T cells which are thereby activated. The activated T cells release a variety of mediator-proteins (lymphokines) independent of the specific antigen initiating the immune response. These lymphokines amplify the response to antigen in a non-specific fashion. One of the lymphokines, Interleukin-2 (IL-2), has the ability to initiate and continue proliferation of activated T helper cells and antigen-primed cytolytic T cells [64]. The influence of CsA on this activation process has been studied extensively in vitro. In the mixed lymphocyte reaction, CsA causes suppression of T helper cell proliferation and cytotoxic T lymphocyte generation associated with inhibition of IL-2 production. Addition of exogenous IL-2 to the allogenic mixed lymphocyte cultures treated with CsA restores the allogenic responsiveness [63, 65, 66]. This finding suggests that although IL-2 production is inhibited, expression of the IL-2 receptor is not influenced by CsA. At the same time there appears to be a relative sparing of T suppressor subpopulations by CsA

[67, 68]. Apparently the balance of immunoregulatory cells is altered by CsA. In addition, the production of other lymphokines including interferon gamma and B cell stimulating and differentiating factors is also inhibited by CsA. The precise site of action of CsA on the T helper cell is yet uncertain. However, it ultimately may influence directly or indirectly the transcription of messenger RNA coding for lymphokines. The synthesis of lymphokines is, as a consequence, inhibited [57]. In conclusion, CsA exerts its immunosuppressive action by inhibition of the synthesis of lymphokines (especially IL-2) by T helper cells aborting the induction of cytotoxic T cells while sparing the suppressor T cell population.

Side effects

Nephrotoxicity and hypertension represent the most frequent and clinically important complications associated with CsA use, and may ultimately define the limits of clinical utility of the drug for longterm immunosuppression. The most obvious sign of nephrotoxicity is an increase in serum creatinine. Renal dysfunction due to CsA was evident from the first clinical trial in humans [69, 70]. Acute nephrotoxicity will be evident immediately or within the first week after transplantation, particularly in kidneys injured due to other causes e.g. hemodynamic instability of the donor, prolonged cold ischemia, anastomosis time (warm ischemia), urinary tract obstruction and administration of nephrotoxic drugs [71]. Probably CsA potentiates ischemic damage (acute renal failure) or delays reconvalescence. Dialysis support is more frequently required during CsA than AZA therapy and in some studies a compromised longterm graft function was revealed [72, 73, 74]. Not always a difference in the incidence of postoperative anuria was found between CsA and AZA treated kidney allograft recipients but when it occurred the anuria was of longer duration under CsA therapy [74, 75]. The differential diagnosis of rejection and nephrotoxicity in this phase largely depends on the allograft biopsy findings [69, 76]. During CsA therapy it is uncommon for rejection to present with the classical signs and symptoms of fever, graft tenderness, swelling and oliguria. Simultaneous administration of drugs with known nephrotoxicity (e.g. aminoglycosides) will cause additive effects and have to be omitted [77]. Also acute nephrotoxic episodes are frequently seen in the first three months after transplantation and are characterized by a generally modest but not progressive rise in serum creatinine occurring in a few days [78]. Dose reduction of CsA almost always ameliorates the toxicity especially when the elevation of serum creatinine is $\leq 25\%$ [79]. Measurement of CsA levels may be helpfull although the severity of dysfunction correlates poorly with dosage or CsA level using the polyclonal RIA [52, 53, 54].
Chronic CsA induced nephrotoxicity is frequent among renal allograft recipients. Patients maintained on chronic CsA therapy have higher stable serum creatinine values than patients on conventional immunosuppressive therapy [71, 72, 73, 75, 80, 81]. Although the administration of CsA is controlled at therapeutic immuno-

suppressive levels, a mild and non-progressive increase in serum creatinine occurs in the majority of cases within a few weeks after initiation of treatment. Dose reduction will only lead to a small decline in serum creatinine and introduces the risk of under-immunosuppression with inevitable rejection [82]. Investigation of CsA associated morphologic changes showed isometric vacuolization and giant mitochondria in proximal tubular cells and sometimes a mild tubular interstitial nephritis [75, 83]. In more severe forms of nephrotoxicity arterial hyalinosis and intimal thickening were found, distinct in distribution from the pattern of vascular rejection and correlated with interstitial fibrosis. Differentiation between mild rejection and chronic nephrotoxicity can be difficult but the pattern of acute rejection is not altered by CsA.

Understanding of the mechanism of CsA induced nephrotoxicity is hampered by the lack of a suitable animal model. Three hypotheses have been proposed to explain the mechanism [57, 84, 85]. First, a toxic action by CsA on the renal tubule is suggested by the histopathological findings of the proximal tubular cell and a primarily tubular lesion could affect glomerular filtration by back pressure along the nephron or by a feedback mechanism decreasing glomerular blood flow. Secondly, a direct toxicity of CsA on the glomerulus may produce a decreased filtration coefficient and glomerular dysfunction, influencing renal haemodynamics and tubular function. The third hypothesis suggests that CsA interferes with the synthesis of prostacyclin stimulating factor, thereby influencing the humoral renal regulation mechanisms and in addition causing endothelial damage of the arterioles with thrombo-embolic complications similar to the haemolytic uremic syndrome [86, 87]. All these postulated actions of CsA can ultimately deteriorate and progressively impair kidney allograft function. The longterm effects of CsA on the kidney are not yet established, but an important question will be the degree of reversibility after stopping CsA.

An interrelationship between nephrotoxicity and hypertension is likely. The persistant elevation of blood pressure often requires intensive combination anti-hypertensive drug regimens. The frequency of hypertension is around 70% in CsA treated renal allograft recipients and shows a significant increase in the Canadian Multicenter trial compared with AZA treated patients [71, 72, 88].

An increase in serum lipid values and a deterioration in carbohydrate metabolism attributable to CsA is reported in renal transplant recipients [89].

The incidence of bacterial, viral and fungal infections is not changed in comparison with AZA treatment but life threatening infections are less frequent [71, 73, 79]. A decrease in the overall amount of immunosuppression due to a lower incidence of rejection and the absence of myelotoxicity will be important in this respect. The occurrence of non-T cell lymphoma in 6 patients in the first pilot studies using CsA in clinical transplantation led to grave concern [70]. Over-immunosuppression was considered as the most important factor and reduction or discontinuation of immunosuppression was sufficient to cause regression [90]. In all patients who developed lymphoproliferative disorders previous Epstein-Barr virus infections

were shown. The combination of immunosuppressive agents used may have favoured an escape from T cell surveillance normally exerted over proliferation of Epstein-Barr virus-infected B cells.
The increased risk of neoplasia appears to represent the risk of broad immunosuppression rather than an unique attribute of CsA. Breast fibro-adenomas are regularly reported in female CsA treated patients [78, 80, 81]. Gingival hyperplasia, clinically similar to that seen with diphenylhydantoin, occurs in 8 to 25% of CsA treated kidney transplant recipients and is reversible [70, 71, 75, 78, 79, 81]. Hirsutism is a frequent but reversible side effect developing shortly after starting CsA and involves predominantly face, arms, eyebrows and back [70, 71, 78, 79, 81]. Tremors and paresthesias of hands (10-40% and 10%, respectively) are responsive to drug dosage lowering [70, 71, 78, 79, 81]. Epileptic seizures are seldom but may occur at very high CsA levels with concommitant hypertension or high dose corticosteroids as permissive factor [81]. Hepatotoxicity is of little clinical significance with currently used dosages. Transient but small elevations in serum bilirubine and transaminase levels attributed to cholestasis have been reported [70, 71, 75, 78, 81]. Minor gastrointestinal symptoms occurring in the first weeks after starting CsA therapy are transient [81].

Clinical results

In 1978 CsA was first used by Calne et al. in clinical transplantation [69]. They showed that CsA was extremely effective in preventing rejection of renal allografts. However, the addition of CsA to other immunosuppressive drugs (cyclophosphamide, corticosteroids, AZA) in the early clinical experiments caused numerous complications, including lymphoma's, fatal infections and impaired allograft function (nephrotoxicity). These findings led to the recommendation that CsA should be used alone [70]. High dose corticosteroids were only added when rejection occurred. Using this protocol the one year graft survival of poorly matched kidneys, though selected for primary function, was 86% [80]. This Cambridge regimen has been used as the basis of the European Multicenter Trial. In this prospective randomized study CsA alone was compared with conventional immunosuppression (AZA and steroids) [81]. One year graft survival was 72% in the CsA treated group and 52% in the control group. At five years, graft survival was 55% in CsA and 40% in AZA treated patients [91]. Remarkably, the subgroup converted from CsA to conventional therapy for various reasons during the first year, achieved only a 60% one year graft survival but the five years survival in these switched patients was 54%. The advantage of this study protocol was a reduced amount of overall immunosuppression and avoidance of steroid induced side effects. Still a significant number of rejection episodes occurred in the CsA treated group necessitating a considerable amount of steroids to reverse rejection.
Another approach was outlined by Starzl et al. [92]. Maintenance low dose steroids were added to CsA from the day of transplantation suggesting a synergistic

immunosuppression as they had shown previously with AZA [29]. The results of this combination therapy were very good. In the initial studies a 80 to 90% one year graft survival was reached [76, 92, 93]. The advantage of this combination regimen was a lower incidence of graft rejection episodes but the patients were still exposed to the potential side effects of longterm steroid treatment and an increased risk of infection and development of lymphoma due to a greater amount of immunosuppression. In other centers similar improvements in graft survival were achieved with this CsA and prednisone (CsA/P) therapy compared to historical control groups [75, 94]. Ultimately CsA/P was compared with AZA and prednisone (AZA/P) in a prospective randomized study (Canadian Multicenter trial). One year graft survival among CsA/P treated allograft recipients was 78% and among AZA/P treated patients 65% [71]. At three years, graft survival was 69% in the CsA/P treated patients and 58% in the controls (AZA/P) [72]. Although the number of rejection episodes was similar in the two groups, they were significantly more severe among controls. However, in several individual centers one year graft survival rates of 70% or more were already achieved using AZA/P [95, 96] and the additional benefit of CsA was not as impressive as in both multicenter trials [97, 98]. But is has to be noted that in some centers ALG was included in the group of AZA/P treated patients during the posttransplantation period and that the recipients were given multiple transfusions and were splenectomized [78, 99, 100].

Clearly, CsA represents an attractive alternative to AZA/P for renal transplantation. Although a significant improvement in allograft survival has not always been shown, the convalescent period posttransplantation has been marked by fewer overall complications, less clinical morbidity, fewer rejection episodes, no greater frequency of infectious complications, shorter hospital stays and last but not least an improved patient survival. Whether CsA has to be used alone or in combination with steroids is still under discussion. However, no difference in one year graft survival has been found comparing CsA with CsA/P [101, 102, 103].

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CHAPTER 2

CONVERSION FROM CYCLOSPORIN A TO AZATHIOPRINE: AIM OF THE STUDY

Cyclosporin A (CsA) has caused an important improvement of the results in kidney transplantation. However, the nephrotoxic potential of the agent was evident since the first clinical studies and impaired renal function was not only observed in kidney transplant recipients but also in patients with other organ transplants who received CsA for immunosuppression [1, 2, 3].
In 1984 a report from Sweden demonstrated that longterm CsA use resulted in interstitial fibrosis and tubular atrophy and subsequently permanent kidney damage. The pathological changes were dose related for they had been seen to progress only in patients who had received a high cumulative dose of CsA during the first six months of treatment [4]. In another morphological study a progression of fibrosis was reported despite optimalization of CsA therapy [5].
Also in 1984 serious renal dysfunction was reported in heart transplant recipients treated with CsA for more than a year, ultimately requiring hemodialysis for end-stage kidney failure in a few patients [3]. Increasingly, reports appeared dealing with a fixed or progressive impairment of renal function in CsA treated kidney recipients [6, 7, 8]. Whether this CsA induced renal dysfunction was reversible or irreversible remained obscure. These findings raised the question whether CsA would prove of longterm benefit in comparison to conventional immunosuppression with azathioprine (AZA) in kidney transplantation. When considering this question one must not forget that CsA yielded higher rates of one year engrafment than AZA.

What manoeuvres might preserve the potent immunosuppressive properties of the drug, while minimizing its nephrotoxicity?
The first strategy is a reduction of the drug dose. After the introduction of blood level monitoring, drug doses were adjusted to achieve concentrations which are consistent with clinical efficacy but which do not cause adverse drug effects. This manoeuvre proved to be especially effective in acute nephrotoxicity. Most side effects are dependent on dose and their incidence is low at the currently used CsA doses. Impaired renal function and hypertension usually improve when the dose of CsA is reduced. However, when CsA treated kidney transplant recipients are compared with a control group receiving AZA therapy, serum creatinine and bloodpressure are still higher despite optimalization of drug dose [6, 7].
A second method of reducing the incidence of CsA induced nephrotoxicity is triple therapy. The concept of triple drug maintenance therapy is based upon the

rationale that the side effects of each drug (particularly the nephrotoxicity of CsA) when administered in low doses, are minimal and that chronic rejection is better controlled by the synergistic (or additive) effect of all three drugs. In such a regimen low doses of AZA and prednisone (if not already prescribed) are added and the dose of CsA is gradually tapered down. One year graft survival was not influenced by this triple drug maintenance treatment compared with CsA alone or CsA and prednisone, and renal function improved [9, 10]. Although in low dose, CsA can still act as a nephrotoxic agent on longterm. In two prospective controlled studies no difference in serum creatinine between CsA alone and CsA in combination with prednisone or in triple therapy has been found on longterm [11, 12].

A third protocol uses CsA only to obtain engraftment. CsA is administered just during the period of immunological high risk estimated at 3 to 6 months after transplantation, followed by switching treatment to alternative conventional immunosuppression with AZA and prednisone [13, 14, 15, 16, 17, 18, 19, 20, 21]. Serum creatinine levels in nearly all patients fell after conversion to AZA at three months after transplantation. This suggests the existence of nephrotoxicity in a large proportion of patients, despite intensive drug monitoring in blood or plasma. However, the switch to conventional immunosuppression 3-6 months after transplantation resulted in a high incidence of graft rejection [13, 14, 15, 16, 17, 18, 19, 21] and the shortterm superiority of the immunosuppressive effect of CsA and corticosteroids might be lost in longterm follow-up studies.

An appropriate concern about the effect on longterm benefit in kidney transplantation and the potential reversibility of CsA induced nephropathy has accompanied the use of CsA in our transplantation center. Optimalization of drug dose by blood level monitoring or using low dose CsA does not exclude the possibility of inducing renal dysfunction whether fixed or even progressive.

In an attempt to minimize the nephrotoxic effect on longterm we decided to convert kidney transplant recipients from CsA to AZA. We delayed conversion to conventional immunosuppressive therapy for a period of 12 months after transplantation because: a. early conversion is complicated by a high incidence of rejection, b. the improved results in graft survival due to CsA are mainly achieved within the first year posttransplantation and c. severe CsA induced nephropathy is reported to occur after more than one year of continuous CsA treatment.

In this thesis the results of late conversion from CsA to AZA therapy on graft survival and the reversibility of CsA related side effects are discussed. We studied 23 kidney transplant recipients who had continuously received CsA during the first postoperative year and investigated the incidence and severity of CsA induced renal dysfunction, hypertension and metabolic disturbances before and 3 months after conversion from CsA to AZA. Both functional and morphological indices were evaluated. We also studied changes in parameters of cellular immune responsiveness resulting from this conversion. In addition, the differential effects of CsA and AZA on the humoral immune responsiveness were investigated in a separate study.

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CHAPTER 3

ENGRAFTMENT AND METABOLIC INDICES

INTRODUCTION

The results of organ transplantation have significantly improved after the introduction of cyclosporin A (CsA). In most centers, the one year graft survival after kidney transplantation in adult patients showed a significant improvement accompanied by a reduced incidence of acute rejection episodes compared to conventional immunosuppressive therapy with azathioprine (AZA). Unfortunately, CsA is nephrotoxic and it has been suggested that any benefit derived from the reduced incidence of rejection in the first few months after transplantation may be more than offset by the progressive and potentially irreversible nephropathy on longterm [1]. Several investigators have embarked on protocols of routine conversion from CsA to AZA at a fixed interval posttransplant to avoid any longterm sequelae of CsA administration [2, 3, 4, 5, 6]. Admittedly, there is a functional reversible form of CsA induced kidney damage in the early period after transplantation, but data on the potential reversibility of renal impairment after CsA has been given for longer periods than 3-6 months, are not available. Early conversion from CsA to AZA therapy at 3 months after transplantation is associated with a substantial incidence of rejection (20-33%) and even graft loss [2, 4, 5]. Delaying conversion for six months has been no more successful, since rejection has followed in the same percentage of patients treated this way [6]. The desire to maintain the improvement in longterm renal allograft survival achieved with CsA as the primary immunosuppressive drug, combined with our concern for potential irreversible nephrotoxicity on longterm led us to the attempt to induce a safer engraftment by continuation of CsA therapy for one year. This chapter describes the fate of 23 cadaveric renal allograft recipients who took part in a conversion protocol one year after transplantation. The potential reversibility of chronic CsA related nephrotoxicity, hypertension and alterations in lipid and carbohydrate metabolism is studied and discussed in relation to the risk of rejection.

PATIENTS AND METHODS

Between November 1983 and August 1984, 41 nondiabetic adult patients received a cadaveric kidney allograft at our center. The standard immunosuppressive

protocol consisted of CsA and prednisone. CsA was started six hours after transplantation at a dose of 4 mg/kg/day given by continuous infusion for the first 36-48 hours. Thereafter the treatment was continued orally, initially in a dose of 14 mg/kg/day given in 2 divided doses. The oral dose of CsA was gradually tapered off aiming at plasma trough levels between 50-150 ng/ml. A commercially available conventional polyclonal radioimmunoassay kit (Sandoz Ltd., Basle, Switzerland) was used to measure CsA plasma levels. Corticosteroids were started immediately after transplantation (400 mg hydrocortisone by continuous infusion over 24 hours) and rapidly reduced to a daily oral dose of 10-15 mg prednisone. Biopsy-proved rejection crises were treated with a 21-day course of rabbit anti-thymocyte globulin (RATG, RIVM, Bilthoven, The Netherlands), administered in 3-6 doses of 4 mg/kg bodyweight in order to keep the circulating T cells between 50-150/mm^3.

During the first year after transplantation 2 patients died and in 6 patients an acute rejection episode resulted in graft loss. The overall actuarial one year graft survival and one year patient survival were 81.0% and 95.3%, respectively. In the first 5 months after transplantation 9 patients were already converted to AZA therapy. In 7 of them difficulties in distinguishing CsA nephrotoxicity from rejection had led to this decision. One patient was switched to AZA because of a severe degree of hirsutism and one patient was converted in another center after three months. Conversion to AZA was contraindicated in another patient who had suffered from peliosis hepatis while receiving AZA during a previous transplant.

The remaining 23 patients, who had received one year continuous CsA therapy, formed our study group. Thirteen of them were male. Their ages ranged from 17-66 (median 48) years. There were 16 recipients of a first transplant and 7 of a second transplant. At the time of conversion all 23 patients had stable kidney function and had not experienced rejection crises in the 6 months before conversion. They were admitted to a metabolic ward and received a diet with a fixed content of sodium (70-90 mmol/day). Compliance was assessed by 24 hour urine collections. Antihypertensive treatment, if any, was continued. None of the patients used non-steroid anti-inflammatory drugs. After sodium balance was achieved and 24 hour urine creatinine excretion became stable, two consecutive 24 hour urine collections bracketed by fasting serum creatinine determinations were taken for calculation of endogenous creatinine clearance. Plasma and urine creatinine concentrations were determined by a modified Jaffé-reaction using an auto-analyzer technique. The standard formula $C_x = U_xV/P_x$ was used for the calculation of clearance where U_x and P_x are the urine and plasma concentration of substance x, C_x its clearance and V is the urine flow rate. After an overnight fast the patients were investigated in the laboratory. Venous blood samples for determination of CsA trough levels and electrolytes were drawn and radiolabelled clearance studies, as discussed in chapter 4, were performed. Blood pressure and heart rate were recorded in semisupine position with an automatic device (Accutorr TMI, Datascope Corp., Paramus, NY, USA). Twelve consecutive readings made at five

minute intervals were averaged. The next day fasting blood samples were taken for determination of cholesterol and triglycerides, and a standard 75 gram oral glucose tolerance test (GTT) was performed. One hour urine collections were checked for glucose by dipstick during oral GTT. Then CsA was stopped and AZA was introduced in a dosage of 2 mg/kg bodyweight. All other medication, including antihypertensive drugs, was continued. Three months after conversion to AZA the patients were readmitted to the hospital for a similar, second evaluation. At the frequent visits to the outpatient clinic after conversion, serum creatinine and creatinine excretion in 24 hour urine collections were determined and blood pressure was recorded. The follow-up period was 18 months. All data are presented as mean ± SEM. Statistical analysis was performed using the Fisher's exact test and the Student's t test for paired data. Statistical significance was accepted at the 95% confidence level.

RESULTS

Before conversion from CsA to AZA

At the time of conversion CsA plasma trough levels were 98 ± 13 ng/ml. The oral dose of CsA was 6.1 ± 0.9 mg/kg bodyweight (b.i.d). Serum creatinine was 172 ± 12 μmol/l and the calculated creatinine clearance 48 ± 3 ml/min. Thirteen patients were on antihypertensive drug treatment. Blood pressure before conversion was systolic 140 ± 3 mmHg and diastolic 89 ± 2 mmHg with a mean arterial pressure of 114 ± 3 mmHg.
The fasting glucose level was 4.5 ± 0.2 mmol/l and rose to 7.2 ± 0.5 mmol/l at two hours after glucose loading. Serum cholesterol and triglycerides were 8.0 ± 0.5 mmol/l and 3.2 ± 0.3 mmol/l, respectively.

After conversion from CsA to AZA

Acute rejections
Within 6 weeks after conversion 5 of the 23 patients (22%) showed a biopsy-proved rejection. Four of them were recipients of a second allograft ($p < 0.01$). Two of the five rejections could only partly be reversed with RATG and 2 patients lost their graft. Both were recipients of a second allograft. Between non-rejecting and rejecting patients no significant differences were found in the number of HLA-A, B and DR mismatches, percentage of peak panel reactive lymphocytotoxic antibodies, number of pretransplant blood transfusions or previous rejection episodes (table I). Serum creatinine, creatinine clearance, actual CsA dosage and CsA plasma trough levels during the preconversion hospital admittance were also non-predictive for outcome after conversion. Of the 7 recipients of a second allograft, 4 rejected their graft (57%) compared with one of the 16 first allograft recipients (6%). Table II shows the comparison between first and second transplant

recipients. No significant differences in the preconversion characteristics were found between these groups. One patient had a recurrence of a membrano-proliferative glomerulonephritis within 3 months after conversion, ultimately requiring haemodialysis therapy. Three months after conversion the remaining 17 patients were readmitted for re-evaluation as described before.

Table I
Preconversion characteristics of patients with and without rejection after conversion.

	No rejection	Rejection
Number of patients	18	5
Male/female	11/7	2/3
First/second transplants	15/3	1/4 *
Number of mismatches HLA-A, B	1.5 ± 0.3	2.2 ± 0.6
HLA-DR	0.6 ± 0.1	0.6 ± 0.4
% Lymphocytotoxic antibodies-median (range)	26 (3-100)	56 (18-61)
Number of pretransplant blood transfusions-median (range)	4 (1-36)	4 (1-21)
Mean number of preconversion rejection episodes	0.3	0.4
CsA dose (mg/kg/day)	6.4 ± 1.1	5.2 ± 0.8
CsA plasma trough levels (ng/ml)	106 ± 16	69 ± 8
Creatinine clearance (ml/min)	46 ± 3	57 ± 7
Serum creatinine (μmol/l)	176 ± 12	161 ± 38

* $p < 0.01$ Fisher's Exact Test

Table II
Preconversion characteristics of first and second transplant recipients.

	First	Second
Number of patients	16	7
Male/female	9/7	4/3
Number of mismatches HLA-A, B	1.7 ± 0.3	1.6 ± 0.4
HLA-DR	0.6 ± 0.1	0.7 ± 0.3
% Lymphocytotoxic antibodies-median (range)	20 (3-88)	54 (18-100)
Number of pretransplant blood transfusions-median (range)	5 (1-36)	4 (1-7)
Mean number of preconversion rejection episodes	0.3	0.4
CsA dose (mg/kg/day)	5.0 ± 0.4	8.6 ± 2.6
CsA plasma trough levels (ng/ml)	91 ± 11	112 ± 37
Creatinine clearance (ml/min)	48 ± 3	49 ± 3
Serum creatinine (μmol/l)	167 ± 13	186 ± 27

Serum creatinine and creatinine clearance

The effect of stopping CsA on serum creatinine in the 17 successfully converted patients is showed in fig. 1. A drop from 171 ± 12 μmol/l to 129 ± 7 μmol/l at three months after conversion was observed ($p < 0.001$). End point values were almost reached at 3 weeks. Fig. 2 shows the rise in creatinine clearance from 47 ± 3 ml/min to 66 ± 4 ml/min ($p < 0.001$). This increase of 40% was also reached within 3 weeks after conversion.

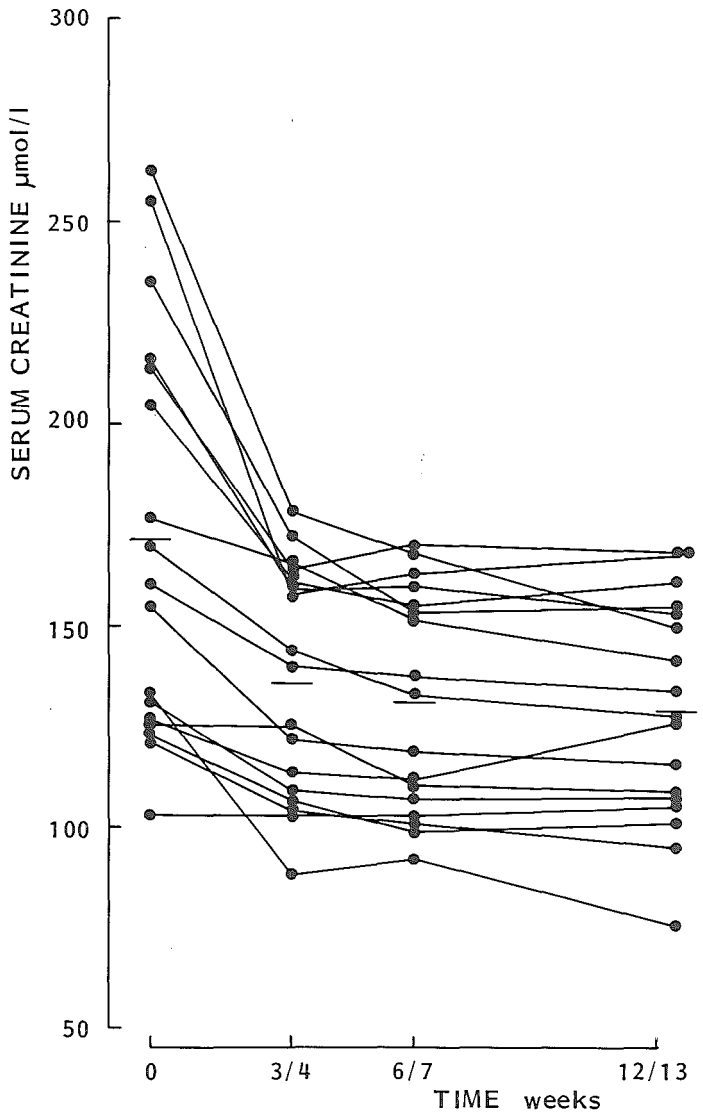

Figure 1 Serum creatinine levels after conversion from cyclosporin A (CsA) to azathioprine (AZA) in the individual patient (n=17).

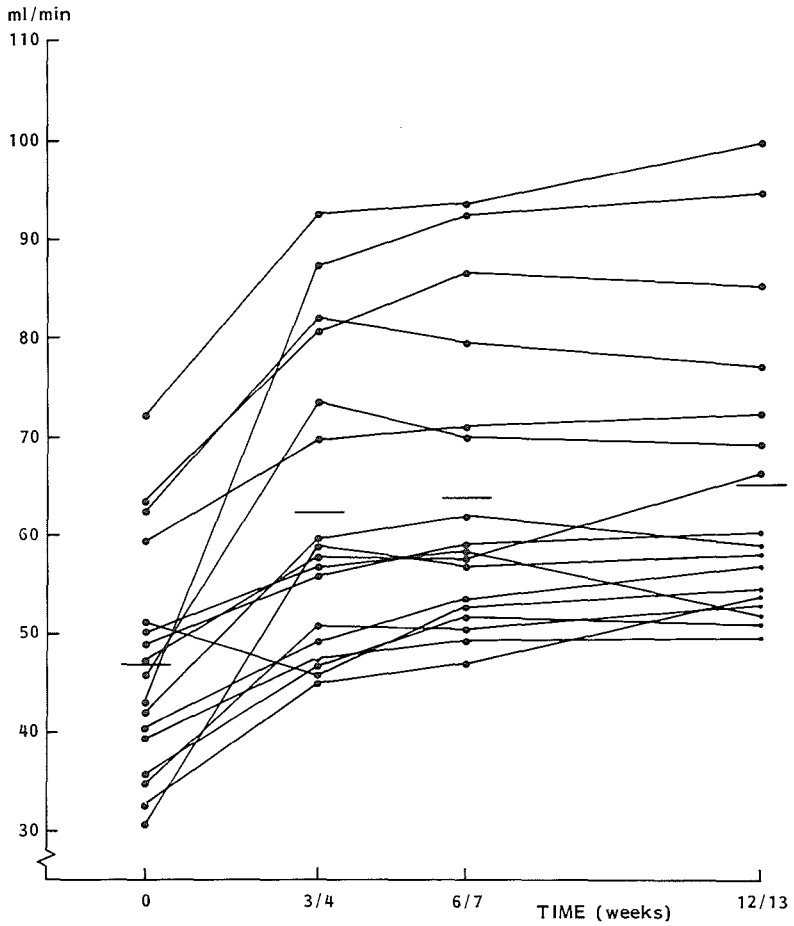

Figure 2 Endogenous creatinine clearance after conversion from cyclosporin A (CsA) to azathioprine (AZA) in the individual patient (n=17).

Blood pressure

Blood pressure fell from systolic 142 ± 4 to 123 ± 3 mmHg ($p < 0.001$) and diastolic from 90 ± 3 to 77 ± 2 mmHg ($p < 0.001$). Three months after conversion mean arterial pressure decreased from 116 ± 4 mmHg before to 97 ± 3 mmHg ($p < 0.001$). Measurements during the visits to the outpatient clinic showed that the decline was most obvious during the first weeks after conversion (fig. 3). The heart rate did not change (62 ± 2 vs. 63 ± 2 beats/min, n.s.).

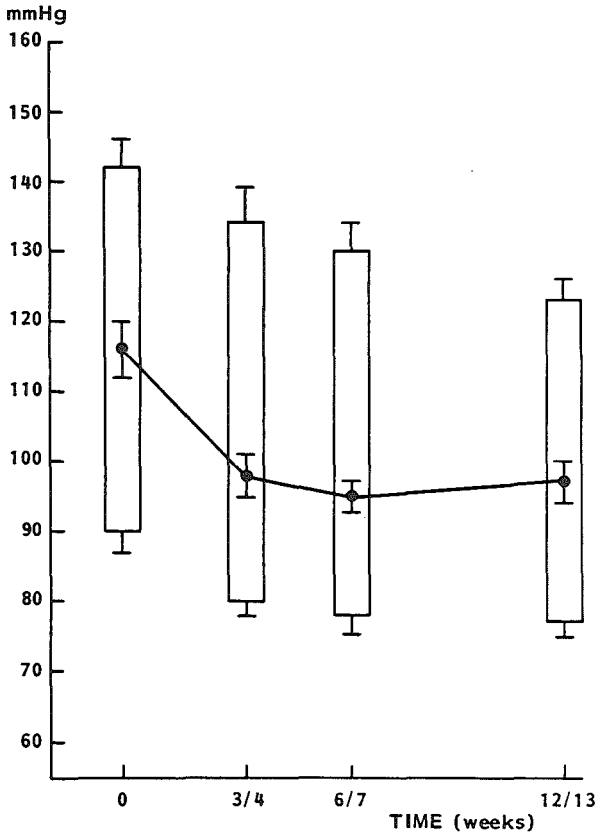

Figure 3 Blood pressure recordings after conversion from cyclosporin A (CsA) to azathioprine (AZA) (mean ± SEM, n=17).

Metabolic changes

After conversion, fasting glucose levels did not change (4.5 ± 0.2 vs. 4.3 ± 0.1 mmol/l, n.s.), but a significant fall was found in glucose concentrations two hours after carbohydrate loading (7.2 ± 0.5 vs. 5.5 ± 0.3 mmol/l, $p < 0.05$, table III). Before conversion, in 5/17 patients glucose level two hours after carbohydrate loading was > 8 mmol/l compared to 0/17 patients postconversion ($p < 0.05$). In no patient glucosuria was found before or after conversion.

In table III it is shown that both serum cholesterol and serum triglyceride levels were significantly lower at 3 months after conversion.

Table III
Metabolic indices before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA) (n = 17, mean ± SEM).

	CsA	AZA
Fasting blood glucose (mmol/l)	4.5 ± 0.2	4.3 ± 0.1
Blood glucose 2 hrs after GTT (mmol/l)	7.2 ± 0.5	5.5 ± 0.5*
Serum cholesterol (mmol/l)	8.0 ± 0.5	6.5 ± 0.4**
Serum triglycerides (mmol/l)	3.2 ± 0.3	2.2 ± 0.2**

* $p < 0.05$ Student's t test
** $p < 0.01$ Student's t test

Follow-up

In the 18 months follow-up period after conversion no additional rejection epsiodes were diagnosed in our patient group. No significant changes, individual nor overall, in serum creatinine determinations and blood pressure recordings were found compared to the postconversion evaluation (fig. 4 and fig. 5).

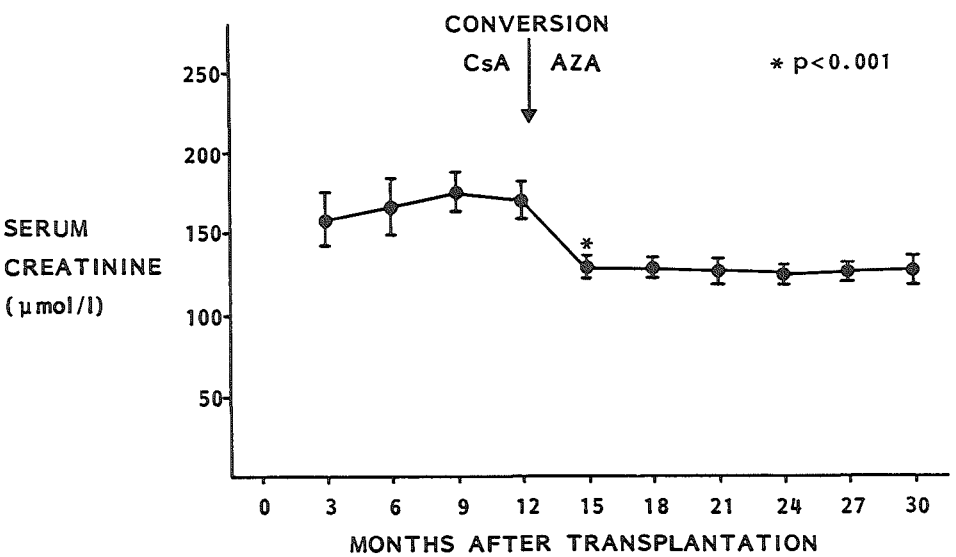

Figure 4 Serum creatinine levels during 30 months after transplantation (mean ± SEM, n=17).

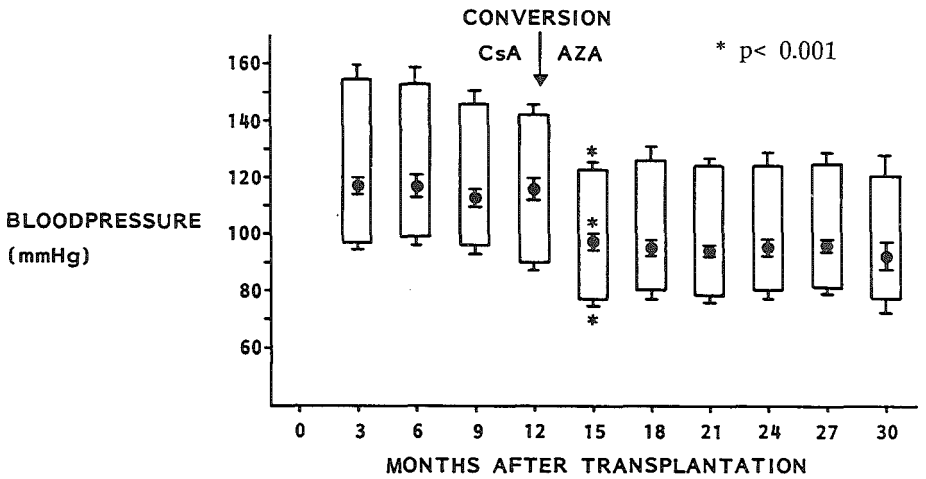

Figure 5 Blood pressure recordings during 30 months after transplantation (mean ± SEM, n=17).

DISCUSSION

In the present study the continuation of CsA therapy for one year after renal transplantation did not result in an overall safer engraftment after switching to AZA. Our incidence of postconversion rejection of 22% is comparable to the results of those centers where conversion was performed after 3 or 6 months CsA treatment [2, 4, 5, 6]. However, from our study it is clear that one group of patients is particularly at risk: recipients of a second allograft. Of 7 patients, 4 rejected their graft as compared with 1/16 of the patients who had received a first allograft. For the latter group conversion from CsA to AZA is a safe procedure. Conversion should be attempted when the benefits of changing therapy outweigh the risks of longterm CsA treatment. One of these risks could be progressive and irreversible renal damage due to an intrinsic nephrotoxicity of CsA, as described by Myers et al. in a group of heart transplant recipients [1]. However, these heart transplant recipients received larger daily dosages of CsA than our patients (7.4 ± 0.9 vs. 6.1 ± 0.8 mg/kg bodyweight) and pre-existent renal dysfunction was not evaluated. After switching to AZA, serum creatinine fell within 3 weeks in all but one of the 23 patients and remained stable in the 17 patients who did not reject their grafts. Creatinine clearance measured at three months after conversion increased by 40%. The remarkable quick improvement in serum creatinine and creatinine clearance together with the substantial fall in mean blood pressure suggests that one year continuous CsA treatment does not result in permanent structural renal damage.

During follow-up after conversion serum creatinine and blood pressure remained stable which is also an argument against progressive and irreversible CsA induced kidney damage.
Switching to AZA after 12 months of CsA therapy normalized serum lipids and glucose tolerance. This phenomenon has already been described in transplant recipients who were converted from CsA to AZA three months after transplantation [7]. Fasting glucose levels are not influenced by conversion. In pancreatic transplant recipients a rise in plasma C-peptide concentration was found after glucose loading during CsA treatment, suggesting that CsA does not affect pancreatic beta cell function adversely but induces insulin resistance [8]. Unfortunately insulin concentrations were not measured. In a recent study CsA treated isolated human islets of Langerhans showed a decreased insulin secretion after incubation with glucose when compared to nontreated islets. This finding suggests a direct effect of CsA on insulin secretion by islets cells [9]. Also the use of steroids can contribute to the abnormal handling of carbohydrates and lipids by promoting insulin resistance. Enhanced clearance of prednisone after stopping CsA can explain the effect on glucose tolerance and also on serum lipids postconversion [10].
In conclusion, we have found an significant improvement in serum creatinine, creatinine clearance and blood pressure after one year of CsA treatment. This also holds true for the effect of CsA therapy on disturbances in carbohydrate and lipid metabolism. Our data do not allow to conclude that longterm CsA therapy will not lead to irreversible structural renal damage. However, from a functional point of view such an adverse effect was not apparent, at least not after one year of continuous CsA treatment, and therefore it cannot be an argument in favour of elective early conversion from CsA to AZA. In recipients of a second allograft this procedure is even contraindicated, because of the high risk of postconversion rejection. However, in recipients of a first transplant, where safe conversion is possible, the sequelae of longterm hypertension, elevated serum lipids, impaired glucose tolerance and the high costs of therapy remain arguments in favour of conversion, despite the reversibility of these side effects after stopping CsA. In patients treated with CsA for other indications, e.g. auto-immune diseases, these factors should also be considered and weighed against the potential beneficial effects of longterm CsA therapy.

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CHAPTER 4

RENAL FUNCTION AND BLOOD PRESSURE

INTRODUCTION

Hypertension is the most frequent complication in recipients of a renal allograft and its frequency has increased even further after the introduction of cyclosporin A (CsA) [1]. Progressive heart and brain damage is the leading cause of death in this patient group. Unfortunately, the mechanisms involved in posttransplantation hypertension are multifactorial and poorly understood. Moreover, many renal allograft recipients have already extensive atherosclerotic or hypertensive vascular disease before transplantation. Diseased native kidneys, steroids, rejection and diminished renal transplant function interact in a complex way and all contribute to the hypertensive state. Whatever its origin, there is a general agreement that decreased renal blood flow is a hallmark of posttransplantation hypertension [2, 3]. After the introduction of CsA, its toxic side effects were quickly recognised [4]. Accumulated evidence indicated that CsA can cause a wide spectrum of renal damage. Depression of glomerular filtration rate and elevation of blood pressure are of major concern [5, 6]. These adverse effects are not restricted to the vulnerable kidney transplant recipient [7] but occur in recipients of heart [8], liver [9] and bone marrow transplants [10, 11] with normal kidneys as well. It has been proposed that CsA induced nephrotoxicity is not strictly due to direct structural damage to the kidney but rather to severe vasoconstriction [12, 13, 14]. This might be a more generalized functional defect, which would explain the increased incidence of hypertension in CsA treated kidney transplant recipients. However, many other causes of hypertension exist in this patient group [15].
In this chapter we report on detailed measurements of renal plasma flow, glomerular filtration rate and blood pressure in 23 cadaver renal transplant recipients who were treated with CsA for one year and were electively converted to azathioprine (AZA) one year after transplantation. We attempted to determine to what extent reversible renal vasoconstriction was involved in the adverse effect on the kidney and to what extent the CsA induced hypertension was reversible.

PATIENTS AND METHODS

Twenty-three nondiabetic cadaveric renal allograft recipients were included in this study. All patients had been on continuous CsA and low dose prednisone therapy for one year. A detailed description of this patient group has been given in chapter 3. At the time of conversion from CsA to AZA renal transplant function was stable

and rejection crises had not occurred in the six months before conversion. All studies were performed in a metabolic ward. The patients used a diet containing 70-90 mmol sodium/day and compliance was assessed by 24 hour urine collections. Antihypertensive treatment in 13 patients was continued. After sodium balance was achieved, two consecutive 24 hour urine collections bracketed by fasting serum creatinine determinations were taken for calculating the endogenous creatinine clearance. Plasma and urine creatinine concentrations were determined by a modified Jaffé-reaction using an autoanalyzer technique. On the day the isotope renal function studies were performed, patients remained supine. At the time of these studies blood was drawn for determination of plasma renin concentration and routine biochemistry. The concentration of enzymatically active renin was measured by the enzyme-kinetic method and radioimmunoassay of angiotensin-I [16, 17].

Effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were measured simultaneously as the clearance of ^{131} I- sodiumiodohippurate (^{131} I-hippuran) and ^{125} I-iothalamate (^{125} I-thalamate), respectively. Following the administration of a priming dose, ^{131} I-hippuran and ^{125} I-thalamate were intravenously infused in one arm at a constant rate. After an equilibration period of 120 minutes three samples were obtained at 30 minute intervals from an indwelling cannula placed in an antecubital vein of the other arm. Plasma levels of ^{131} I-hippuran and ^{125} I-thalamate were measured in a gamma scintillation counter, using different counting windows. The mean value of the three samples was used for calculating the clearance. The clearance of a substance x can be calculated by the standard formula $C_x = (U_x V)/P_x$, where U_x and P_x are the urine and plasma concentrations of substance x, C_x is its clearance and V is urine flow rate. When a substance that is neither formed nor metabolized in the body and that is exclusively excreted by the kidney, is infused at a constant rate, $U_x V$ per unit time at steady state is equal to the amount of substance that is infused. Thus $C_x = (I_x V_i)/P_x$, where I_x is the concentration of x in the infusion fluid and V_i the infusion rate. The calculated clearances of ^{131} I-hippuran (ERPF) and ^{125} I-thalamate (GFR) were corrected for a standard body surface area (BSA) of 1.73 m^2. Effective renal blood flow (ERBF) was calculated by dividing ERPF by (1-venous hematocrit). During the equilibrium period of the isotope studies, blood pressure and heart rate were recorded by an automatic device (Accutorr TMI, Datascope Corp., Pyramus, USA). Twelve consecutive readings made at five minute intervals were averaged. Renal vascular resistance was calculated as integrated mean arterial pressure (measured by the Accutorr) divided by ERBF and expressed as dyn.s.cm^{-5}. After the renal function studies had been completed, CsA was stopped and AZA was started in a dose of 2 mg/kg/day. All other medication (prednisone, antihypertensive drugs) was continued without changing the dose.

Three months after conversion the patients were readmitted to the metabolic ward for a similar second evaluation. In the meantime patients were regularly seen at our outpatient clinic to check blood pressure, body weight and serum creatinine.

Data are presented as mean ± SEM. Statistical analysis was performed using the Student's t test for paired data.

RESULTS

Renal function and blood pressure before conversion from CsA to AZA.

After one year of continuous CsA therapy blood pressure was 140 ± 3/89 ± 2 mmHg. Thirteen patients were on antihypertensive therapy but of these patients three were still hypertensive (diastolic blood pressure ≥ 95 mmHg). Of the ten untreated patients four were hypertensive. Plasma trough levels of CsA were 98 ± 13 ng/ml which is within the therapeutic range. Serum creatinine was 172 ± 12 μmol/l and creatinine clearance was 48 ± 3 ml/min. ERPF and GFR were 193 ± 9 ml/min and 47 ± 3 ml/min, respectively.

Renal function and blood pressure after conversion from CsA to AZA

As described in chapter 3, five patients had a biopsy-proved rejection episode within six weeks after conversion from CsA to AZA. One patient had a recurrence of her membrano-proliferative glomerulonephritis. These six patients were not included in our analysis of the effects of conversion on renal function and blood pressure. The clinical characteristics of the remaining 17 successfully converted patients are summarized in table I.

Table I
Clinical preconversion characteristics of renal allograft recipients successfully converted to azathioprine after one year of continuous cyclosporin A therapy.

Patient no	Age (yr)	Sex	Weight (kg)	Blood pressure (mmHg)	Antihypertensive therapy
1	66	m	75	167/93	atenolol 100 mg, nifedipine 40 mg
2	43	m	90	151/102	
3	44	m	72	156/105	
4	41	m	104	146/99	atenolol 100 mg
5	34	m	78	135/83	atenolol 100 mg, furosemide 40 mg
6	31	m	67	136/86	atenolol 100 mg
7	48	m	76	162/102	
8	52	m	86	147/91	propanolol 320 mg
9	65	m	105	135/84	atenolol 100 mg
10	63	f	56	117/75	isosorbidedinitrate 80 mg
11	51	m	93	133/91	
12	54	f	77	113/66	atenolol 100 mg, furosemide 40 mg
13	54	f	65	133/74	atenolol 100 mg
14	54	f	70	132/81	alpha-methyldopa 1000 mg
15	42	f	55	140/90	
16	35	m	75	151/101	atenolol 100 mg, nifedipine 40 mg
17	40	f	79	168/106	atenolol 100 mg, nifedipine 40 mg

In three weeks after conversion from CsA to AZA, serum creatinine had decreased from 171 ± 12 to 136 ± 7 μmol/l ($p < 0.001$, fig. 1) and remained constant thereafter. This was accompanied by a significant decline in mean arterial blood pressure from 116 ± 4 to 98 ± 3 mmHg. Heart rate did not change.

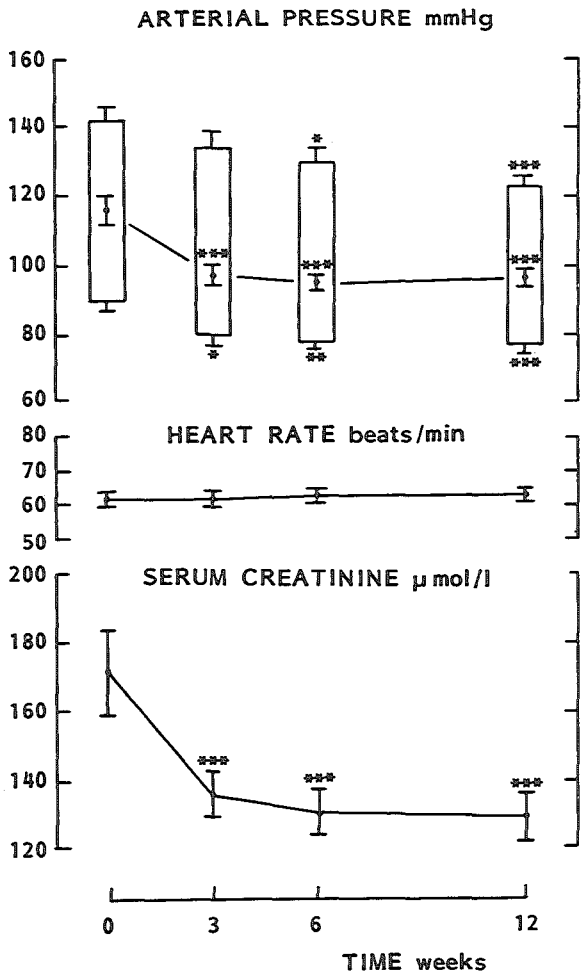

Figure 1 Changes in blood pressure, heart rate and serum creatinine after conversion from cyclosporin A to azathioprine therapy in 17 renal transplant recipients.
(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. preconversion values).

At the second evaluation, three months after CsA had been stopped, serum creatinine was 129 ± 7 μmol/l and mean arterial pressure had dropped by 16 ± 3% ($p < 0.001$, table II). Renal function studies showed an increase in ERPF by 21 ± 4% ($p < 0.01$, fig. 2). Mean haematocrit did not change and ERBF increased from 328 ± 22 to 380 ± 27 ml/min (18 ± 6%, $p < 0.01$). Calculated renal vascular resistance decreased from 27710 ± 2070 before to 20430 ± 1650 dyn.s.cm^{-5} (22 ± 6%, $p < 0.01$) after conversion.

Table II
Mean arterial pressure, effective renal plasma flow and venous hematocrit in renal allograft recipients during cyclosporin A (CsA) therapy and three months after conversion to azathioprine (AZA) therapy.

Patient	Mean arterial pressure (mmHg)		Effective renal plasma flow (ml/min)		Hematocrit %	
no	CsA	AZA	CsA	AZA	CsA	AZA
1	141	124	150	179	45	39
2	124	108	122	123	32	39
3	130	97	223	250	40	22
4	129	91	137	191	36	44
5	102	96	216	260	48	33
6	108	88	226	320	34	37
7	139	101	172	236	53	39
8	114	93	138	172	35	40
9	105	89	151	209	40	38
10	94	91	151	192	38	43
11	111	90	239	280	41	40
12	92	97	241	208	50	36
13	113	110	213	228	33	34
14	105	89	179	220	40	40
15	107	75	292	388	37	40
16	135	121	214	212	45	32
17	129	101	201	261	44	36
mean	116	97	192	231	41	37
± SEM	4	3	11	15	1	1
	$p < 0.001$		$p < 0.01$		n.s.	

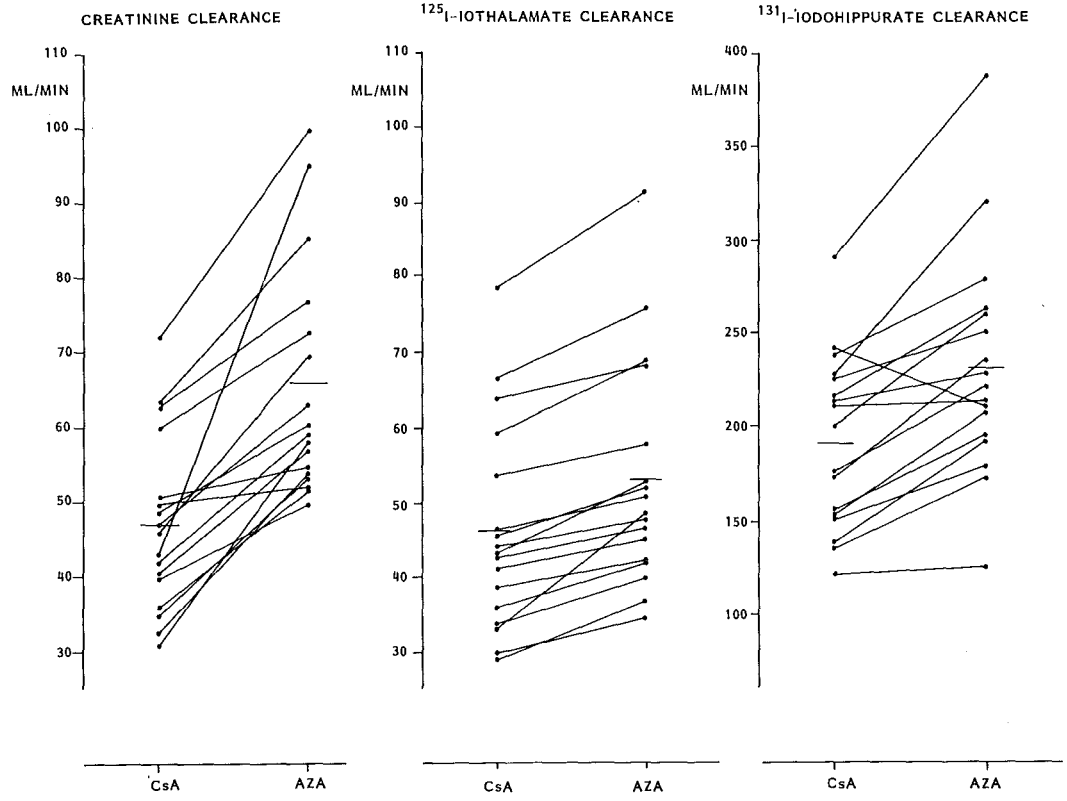

Figure 2 Creatinine clearance, ^{125} I-iothalamate clearance (GFR), ^{131} I-iodohippurate clearance (ERPF) in kidney allograft recipients before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA) therapy.

During CsA treatment the clearance of creatinine did not differ from the clearance of ^{125} I-thalamate (table III) and the calculated creatinine-to-thalamate clearance ratio was 1.04 ± 0.02. After conversion the increase of creatinine clearance (43 ± 7%) was significantly greater ($p < 0.05$) than the increase of ^{125} I-thalamate clearance (16 ± 2%). This resulted in a creatinine-to-thalamate clearance ratio of 1.27 ± 0.06 during AZA treatment which was significantly higher than before conversion ($p < 0.05$).

Table III
Serum creatinine and glomerular filtration rate in renal allograft recipients during cyclosporin A (CsA) therapy and three months after conversion to azathioprine (AZA) therapy.

Patient	Serum creatinine (μmol/l)		Creatinine clearance (ml/min)		^{125} I-thalamate clearance (ml/min)	
no	CsA	AZA	CsA	AZA	CsA	AZA
1	216	153	35	53	34	40
2	235	155	36	51	33	49
3	205	161	42	59	39	42
4	262	150	33	54	29	36
5	255	168	46	70	43	52
6	121	95	72	100	79	91
7	155	116	60	73	59	69
8	215	168	31	58	29	34
9	170	128	43	95	45	52
10	103	105	40	50	36	42
11	123	101	64	85	66	76
12	127	126	50	52	44	47
13	126	109	50	54	53	57
14	177	142	40	57	41	45
15	133	76	47	66	43	46
16	160	134	49	60	46	50
17	132	108	64	77	64	69
mean	171	129	47	66	46	53
± SEM	12	7	3	4	3	4
	$p < 0.001$		$p < 0.001$		$p < 0.01$	

In fig. 3 the creatinine clearance is plotted as a function of the ^{125} I-thalamate clearance. Provided the ^{125} I-thalamate clearance represents true GFR, the fractional clearance of creatinine (creatinine clearance/GFR) increased by 28 ± 6% ($p < 0.01$) after conversion to AZA. The total amount of creatinine excreted per kg body mass per 24 hour during CsA therapy (16.2 ± 0.8 mg/kg/day) was not

significantly different from that excreted during AZA treatment (17.7 ± 0.8 mg/kg/day). Filtration fraction, taken as the ratio between ^{125} I-thalamate and ^{131} I-hippuran clearance, was 0.24 ± 0.01 during CsA treatment and was not changed by switching to AZA (0.24 ± 0.02).

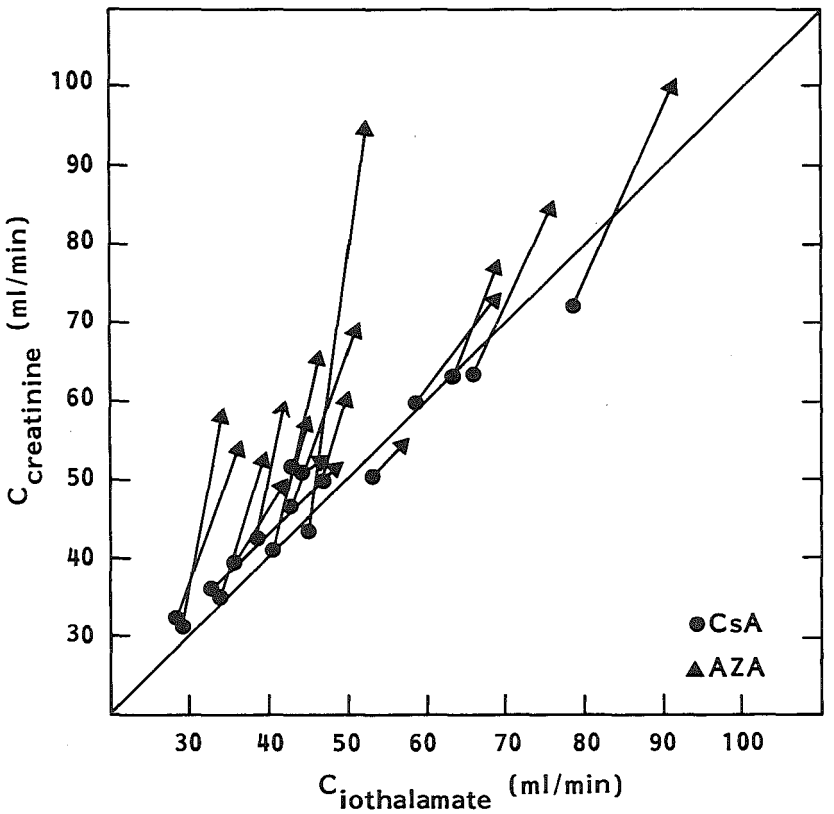

Figure 3 Individual creatinine clearance plotted as a function of the ^{125} I-iothalamate clearance. Circles denote values during cyclosporin A (CsA) therapy, arrows denote values during azathioprine (AZA) therapy and the connecting lines represent the change in the individual patient.

Because plasma renin levels were not distributed normally, log transformation was performed. The geometric mean plasma active renin concentration during CsA treatment was 14.1 μU/ml and increased to 24.4 μU/ml after conversion to AZA ($p < 0.01$, fig. 4). Body weight did not change after conversion (76.4 ± 3.6 vs. 76.7 ± 3.3 kg). The amount of sodium excreted during both three day stays in the metabolic ward was not different (92 ± 6 vs. 105 ± 7 mmol/day).

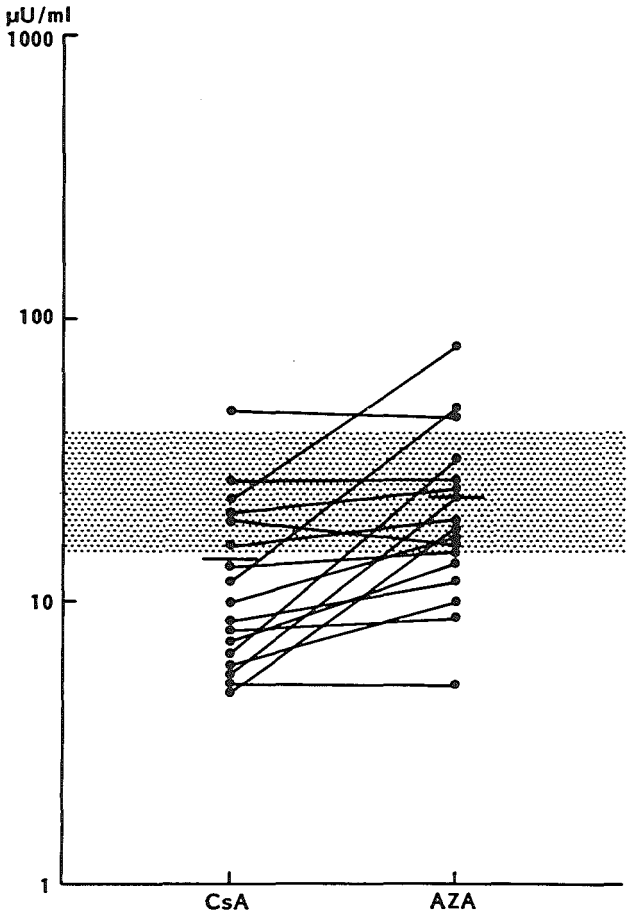

Figure 4 Plasma renin concentration during cyclosporin A (CsA) and azathioprine (AZA) treatment; the dotted area represents the normal range of plasma renin levels.

DISCUSSION

The mechanisms of CsA induced nephrotoxicity are poorly understood. Current thinking is focussed on 1) early, functional toxicity resulting from alterations in renal haemodynamics and 2) late, structural toxicity associated with morphological alterations of tubules and the tubulo-interstitial compartment [6, 12]. Indeed, evidence is accumulating that renal impairment in the early phase of CsA administration is due to a dose dependent renal vasoconstriction [18, 19]. This picture is characterized by a decrease in urine output, an increase in serum creatinine and renal sodium retention [14]. Restoration of renal function follows dose reduction

or discontinuation of the drug. Renal impairment after prolonged CsA administration appears to be characterized by the development of diffuse interstitial fibrosis [20, 21, 22, 23]. Renal failure at this stage is irreversible, at least in part [8]. Which form of nephrotoxicity, functional or structural, was involved in our patients? Our results showed that conversion caused a significant decrease in serum creatinine levels and an increase in ERPF and GFR. These findings suggest that even after long exposure and at therapeutic drug levels, CsA exerts an, at least partially, reversible renal vasoconstrictor effect. These data are in agreement with the findings of Curtis et al., who reported a pronounced renal vasodilatation after withdrawal of CsA [24].

In clinical settings endogenous creatinine clearance is often used as an approximate measure of GFR on the assumption that creatinine at normal serum levels is filtered only. However, creatinine is also secreted by the tubulus and this leads to an overestimation of GFR [25, 26]. This overestimation becomes more important in cases where GFR is decreased. Recently, Tomlanovich et al. have emphasized that these limitations of creatinine in quantifying the severity of loss of GFR are not restricted to renal disease per se but also apply to CsA nephrotoxicity [27]. In our patients the difference between creatinine clearance and ^{125} I-thalamate clearance during CsA treatment was not so pronounced as found by Tomlanovich et al. in their heart transplant recipients, using inulin as "golden standard" for GFR. This could be due to the less compromised GFR in our patients or to a difference in measuring creatinine clearance, two 24 hour periods in our study vs. four 20 minute periods in the study of Tomlanovich et al. More important, however, than the difference in base line values, in our opinion, is our finding that after conversion from CsA to AZA the creatinine clearance rose more than the ^{125} I-thalamate clearance. This indicates that the fraction of creatinine cleared by tubular secretion increased after conversion from CsA to AZA. This can be explained by a direct effect of CsA on tubular secretion mechanisms for creatinine. The practical implication of this finding is that the effect of CsA on GFR can be overestimated when using serum creatinine and creatinine clearance as a criterion. Our findings underscore the plea of Myers and associates to monitor GFR by other means than by creatinine alone [8].

Plasma renin levels in our patients rose after conversion from CsA to AZA confirming other studies in human which have suggested that the renin-angiotensin system is suppressed by CsA [28, 29, 30]. In the present study a high incidence of hypertension was noted during CsA treatment. Conversion from CsA to AZA caused a rapid decline in blood pressure, again suggesting a functional reversible influence of CsA. The possibility of a low renin volume-dependent type of hypertension has to be considered but there was no change in bodyweight after CsA had been stopped. It seems likely that CsA induces vasoconstriction not only in the kidney but also elsewhere in the body. It is not known whether this is a direct effect of CsA but the effect appears functional rather than structural and it is reversible even after longterm CsA treatment.

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CHAPTER 5

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

INTRODUCTION

After kidney transplantation the adverse effect of cyclosporin A (CsA) on allograft function is difficult to assess as other factors such as infection, urinary tract obstruction, recurrence of original nephropathy and rejection may also result in compromised renal function. Although the clinical presentation and prognosis of CsA induced functional nephrotoxicity have been described previously there is only scarce mention of its morphologic concomitants [1].
Several histopathological studies on biopsy specimens of kidney allografts with acute dysfunction have been performed in an attempt to differentiate between rejection and nephrotoxicity induced by CsA [2, 3, 4]. A number of lesions seemed to be associated with the drug: giant mitochondria and isometric vacuolisation of proximal tubular cells [5, 6, 7], arteriolar hyalinosis and intimal thickening [5, 6], mononuclear infiltrates [2] and interstitial fibrosis with tubular atrophy [6, 8, 9, 10]. These lesions can also be found in other conditions and are not strictly diagnostic for CsA nephrotoxicity [11].
As outlined before, in a prospective trial we studied the potential reversibility of CsA nephrotoxicity in cadaveric kidney allograft recipients who had changed from CsA to azathioprine (AZA) one year after transplantation. Renal biopsy specimens were taken before and three months after conversion, irrespective of graft function. In this chapter we report on the incidence and potential reversibility of histopathological lesions associated with CsA after one year of continuous treatment in patients with stable graft function. In addition, the morphological characteristics after conversion to AZA are discussed.

PATIENTS AND METHODS

Clinical data

Biopsy specimens were taken from twenty kidney allograft recipients. These patients took part in the CsA-AZA conversion protocol one year after transplantation. All patients were admitted to our metabolic ward for evaluation of renal function (see chapter 3 and 4 for protocol). On the last day a renal biopsy was performed and CsA was stopped. The next day AZA was started (2 mg/kg/day), while continuing low dose prednisone. Three months after conversion renal eva-

luation was repeated including a biopsy specimen taken from those patients who had successfully converted.

Biopsy procedure

Percutaneous Tru-cut needle biopsy was performed guided by ultrasound picture. The procedure was done only after coagulation variables were found to be adequate.

Biopsy processing

Part of the biopsy tissue was fixed in 4% formaldehyde and embedded in paraffin wax and processed for light microscopic examination. Serial 2 μm thick sections were treated with haematoxylin-azafloxin, Jones, Jones Azan and CAB. Part of the biopsy specimen was snap frozen in liquid nitrogen. Cryostat sections were examined by using commercial monospecific rabbit antisera directed against human IgG, IgM, IgA, IgE, C3, C1Q, fibrinogen and albumin. A control section was incubated with normal rabbit serum.

Biopsy interpretation

All stained sections were coded and examined by two observers who were unaware of the treatment that each patient had received at the moment of biopsy. Adequacy of the biopsy was judged by the number of glomeruli present. The following features were examined by light microscopy.
Glomeruli: mesangial matrix increase; thickening of basement membrane; extra- or intracapillary proliferation (diffuse or focal); glomerular thrombi; glomerular sclerosis; pronounced juxtaglomerular apparatus; periglomerular fibrosis (thickening of Bowman's capsule).
Tubules: epithelial cell vacuolisation (isometric, anisometric) or eosinophilic degeneration; giant mitochondria; tubular casts consisting of protein, erythrocytes, or leucocytes; tubular microcalcifications; tubular basement membrane changes; tubular dilatation and atrophy.
Arterioles (small vessels with a single layer of smooth muscle cells): endothelial cell swelling; insudative lesions (subendothelial eosinophilic fibrinoid deposits); intimal fibrosis; medial hyperplasia (symmetrical or asymmetrical).
Arteries: insudative lesions; hyalinosis; endovasculitis.
Peritubular venules: dilatation.
Interstitium: infiltrates classified as diffuse or focal consisting of mononuclear or polynuclear (neutrophilic and eosinophilic) cells; tubulitis (invasion of mononuclear cells within tubular epithelium); vasculitis (mononuclear cells infiltrating the vessel wall); fibrosis classified as diffuse, focal, stripe-like or periglomerular; oedema; interstitial hemorrhage; calcifications.
The histopathological features were scored on a semiquantitative scale 0/absent, +/mild, ++/moderate and +++/severe. When appropriate only presence (+) or absence (0) was tabulated.

Immunofluorescent microscopy not only recorded the presence or composition of immune deposits, assessed as linear or nodular, but also their location at the glomerular or tubular basement membrane, mesangium and arteriolar wall. Grading was performed according to the intensity of immunofluorescence staining on a semiquantitative scale (0 to +++).
Statistical analysis was performed using the chi-square test, contingency tables and the Student's t test for paired data.

RESULTS

Clinical data

In 16/20 patients elective conversion from CsA to AZA was successfully performed, showing an improvement in renal function without signs of rejection. In two a second biopsy specimen taken at the time of re-evaluation was omitted because of a prolonged bleeding time. Consequently a renal allograft biopsy before and three months after conversion was performed in 14 patients with a stable graft function. The CsA trough concentrations before conversion were within the therapeutic range (101 $\pm$ 22 ng/ml). Serum creatinine decreased in all 14 patients from 171 $\pm$ 13 μmol/l before to 133 $\pm$ 7 μmol/l three months after conversion ($p < 0.001$) and calculated creatinine clearance rose from 47 $\pm$ 3 ml/min to 65 $\pm$ 4 ml/min ($p < 0.001$). In this patient group ^{125} I-thalamate clearance increased from 46 $\pm$ 4 ml/min to 53 $\pm$ 4 ml/min ($p < 0.01$).

Morphological data before conversion

Sufficient biopsy material was obtained in all fourteen patients (median number of glomeruli 14, range 5-30). Glomerular changes other than mild increase in mesangial matrix and periglomerular fibrosis were uncommon before conversion one year after continuous treatment with CsA (table I). Glomerular sclerosis (> 10%) was seen in 2 patients. In four biopsies a prominent juxtaglomerular apparatus was found.
Tubular lesions after one year of CsA therapy were predominantly located in the proximal epithelial cells (table II). Four biopsy specimens showed isometric vacuolisation. A variable degree of anisometric vacuolisation (6 patients) and eosinophilic degeneration (4 patients) were also found; in 3/14 patients cytoplasmatic changes were not observed. No giant mitochondria were identified after special CAB staining. Scattered protein casts were often seen in the tubular lumen. In 3 patients red cell casts were found. Tubular microcalcifications occurred in two biopsies. Thickening of basement membrane and tubular atrophy with concomitant dilatation was present in most specimens. The degree of tubular atrophy corresponded with the extent of interstitial fibrosis ($p < 0.05$).

Table I
Histopathological features of glomeruli before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA).

	CsA	AZA
increase in mesangial matrix	8	10
thickening of basement membrane	3	4
intracapillary proliferation	3	3
extracapillary proliferation	0	0
glomerular thrombi	0	1
glomerular sclerosis (> 10%)	2	0
pronounced iuxtaglomerular apparatus	4	2
periglomerular fibrosis	12	14

Table II
Histopathological features of tubuli before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA).

		CsA	AZA
proximal tubular cells			
cytoplasmatic changes		11	11
isometric vacuolisation		4	0 *
anisometric vacuolisation		6	8
eosinophilic degeneration		4	9
giant mitochondria		0	0
casts			
protein		12	12
erythrocytes		3	3
leucocytes		0	0
microcalcifications		2	3
thickening of tubular basement membrane		8	9
dilatation and atrophy	0	2	1
	+	10	12
	++	2	1

* $p < 0.05$

In most patients the arterioles showed endothelial cell swelling that was associated with mild to moderate arteriolar intimal fibrosis (50%, table III). Pronounced insudative lesions were found in 5/14 patients, all of whom had a serum creatinine concentration of more than 150 μmol/l ($p < 0.05$, table IV). Medial hyperplasia, either symmetrical or asymmetrical, was noted in half our patients. In three a severe form of medial hyperplasia with an onion-like appearance of the vessel wall was present. No intravascular thrombi were found. The arteries showed a mild to moderate hyalinosis (table III). Peritubular venules were dilated in four patients, but no mononuclear cells were seen inside (table III).

Table III
Histopathological features of arterioles, arteries and peritubular venules before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA)

		CsA	AZA
arterioles			
endothelial cell swelling		10	12
insudative lesions		5	2
intimal fibrosis	+/++	5	6
	+++	1	2
medial hyperplasia	+/++	6	7
	+++	1	0
	onion-like	3	0
arteries			
insudative lesions		0	0
hyalinosis	+/++	10	9
	+++	1	1
endovasculitis		0	0
peritubular venules			
dilatation		4	6

Table IV
Relation between insudative arteriolar lesions and serum creatinine after one year of cyclosporine A (CsA) therapy.

serum creatinine (μmol/l)	insudative lesions		
	-	+	
≤ 150	5	0	*
> 150	4	5	

* $p < 0.05$

The interstitium was the last part of the kidney examined (table V). In 13 patients interstitial cellular infiltrates were present. They were focal and not extensive. In these infiltrates mononuclear cells predominated with a small admixture of neutrophilic and eosinophilic polymorphs. In two patients a minimal infiltration between epithelial tubular cells was seen but no invasion of the arteriolar walls was noted. No relationship could be found between graft function and extent or intensity of infiltration. Interstitial fibrosis was present in all biopsy specimens and ranged from mild to moderate. A segmental occurrence of the fibrosis caused a striped appearance in four specimens. The amount of fibrosis was associated with the extent of tubular atrophy ($p < 0.05$). No relationship could be found between the extent of fibrosis and graft function. Interstitial calcifications were present in three patients.

Table V
Histopathological features of the interstitium before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA).

		CsA	AZA	
cellular infiltrates				
intensity	0	1	0	*
	+	6	1	*
	++	7	10	*
	+++	0	3	*
extension	focal	13	11	
	diffuse	0	3	
composition	mononuclear cells	13	14	
	polynuclear cells	3	4	
	eosinophilic cells	0	1	
tubulitis	0	12	5	*
	+	2	6	*
	++/+++	0	3	*
vasculitis		0	1	
fibrosis				
intensity	0	0	0	
	+	7	6	
	++	7	8	
extension	focal	7	7	
	diffuse	3	4	
	stripe-like	4	3	
oedema		0	1	
interstitial hemorrhage		0	0	
calcifications		3	2	

* $p < 0.01$

Before conversion circular nodular deposits of IgM and complement (C3, C1q) were prominent in the arteriolar walls of 12 patients treated with CsA (table VI). The immunofluorescence staining characteristics of glomeruli and tubuli were nonspecific and weak.

Table VI
Immunofluorescence staining characteristics of arteriolar wall before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA).

		CsA	AZA	
arteriolar deposits				
IgM	0	2	8	*
	+	9	4	*
	++	2	2	*
	+++	1	0	*
complement (C3/C1q)	0	1	5	*
	+	4	6	*
	++	5	3	*
	+++	4	0	*

* $p < 0.05$

Morphological data after conversion

Three months after conversion from CsA to AZA therapy a second biopsy was performed and the histopathological findings were compared with those by light microscope before conversion. No changes in appearance of the glomeruli were observed (table I).
Isometric vacuolisation of the proximal tubular cells, present in 4/14 biopsy specimens before conversion, was not found after conversion ($p < 0.05$, table II). No significant changes in anisometric vacuolisation, eosinophilic degeneration and other tubular morphological features were observed.
In two of the 5 patients with arteriolar insudative lesions before conversion, showed the same lesions three months after changing regimens (n.s., table III). No changes in the incidence of endothelial cell swelling or intimal fibrosis were noted. An onion-like appearance of the arteriolar media present in 3/14 biopsy specimens before conversion was not found after conversion. The symmetrical pattern of medial hyperplasia became asymmetrical. The light microscopic appearance of the arteries and the peritubular venules was not changed after conversion (table III). A significant increase in the size of the cellular infiltrates ($p < 0.01$, fig.1), characterized by an increased amount of mononuclear cells ($p < 0.05$) was present in the second biopsy specimens (table V). The location of the infiltrates was still focal in eleven but had become diffuse in three patients. An increased invasion of

mononuclear cells between tubular epithelial cells showing a pronounced tubulitis, occurred in 9/14 postconversion biopsy specimens ($p < 0.01$). A local infiltration of mononuclear cells in the media and intima of arterioles (vasculitis) was present in one patient. The pattern of interstitial fibrosis remained the same after conversion as were the other interstitial features.

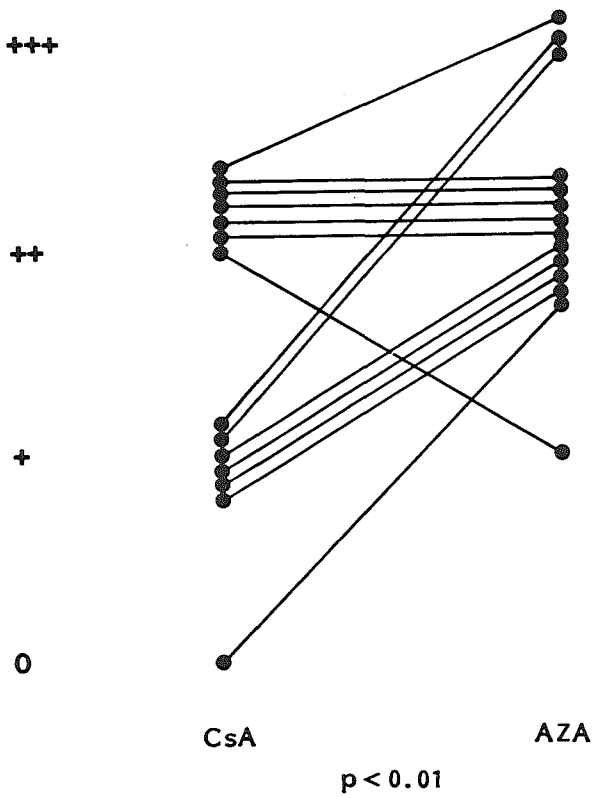

Figure 1 The intensity of the cellular infiltrates before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA), o/absent, +/mild, ++/moderate, +++/severe

Three months after conversion the arteriolar IgM and complement deposits were absent in 7 and significantly less in 2 biopsy specimens in contrast to the 12 specimens before conversion which showed a prominent immunofluorescence staining ($p < 0.05$, table VI). No association with the morphological findings of tubulitis or vasculitis was found.

The improvement in renal function in the individual patient was not related to any specific histologic feature. During a follow-up period of 18 months after conversion there were no rejection episodes.

DISCUSSION

A recent editorial stated that renal biopsy specimens are far more important in ruling out rejection than determination of drug concentrations in the management of suspected CsA nephrotoxicity [12]. Several histopathological studies attempted to define the diagnostic characteristics of this toxicity in renal allografts [2, 4, 13]. Most of these reports were of limited value because CsA treated allografts with normal stable function were not included. When no rejection pattern was evident during periods of renal dysfunction, pronounced and unusual histological features were associated with CsA treatment [6]. After reducing the CsA dose renal function improved, but biopsies were not repeated to evaluate the influence on morphological features and their potential reversibility.

In the present study after one year of continuous and monitored treatment with CsA renal allograft biopsy specimens in patients with stable graft function did not show diagnostic histological markers of CsA nephrotoxicity, but several morphologic features were found to be indicative of compromised renal function induced by CsA [5] and their course after conversion was recorded.

After one year of CsA therapy typical lesions included isometric vacuolisation of proximal tubular cells and arteriolopathy with an almost diagnostic IgM/C3-C1q immunofluorescent pattern. The morphologic picture of tubular isometric vacuolisation is due to dilatation of the smooth and rough endoplasmatic reticulum and identical with osmotic nephrosis. All tubular cells in a cross section contain densely packed empty (free of lipids) vacuoles of equal size. Isometric vacuolisation must be delineated from the vacuolisation found in ischaemic renal damage such as acute tubular necrosis and vascular rejection. The latter shows vacuoles of unequal size with shedding of the cell apex [5, 6]. Proximal tubular isometric vacuolisation was found in four specimens before conversion but not after conversion [5]. No giant mitochondria were identified in our series despite specific CAB staining [7, 14]. Using lower doses of CsA, these tubular lesions are less commonly observed and are therefore probably dependent on dose [5, 6]. The vascular changes after one year CsA therapy were limited to the arterioles. The light microscopic examination showed medial cell hyperplasia and endothelial cell swelling, together with intimal fibrosis in 50% of patients. Pronounced insudative lesions were limited to patients with an increased serum creatinine concentration. Our findings agreed with the recent observation that late biopsy specimens showed a higher incidence of arteriolopathy in CsA treated patients than those treated with AZA [15]. In a recent study, routine renal biopsy specimens in patients with a stable function treated with CsA showed arteriolar medial hyperplasia and arteriolar hyalinosis one month after transplantation [4, 16]. However, these lesions were

less prominent than those in patients with renal dysfunction due to CsA nephrotoxicity at the same time after transplantation. The pathogenesis of arteriolar disease associated with CsA treatment is not yet known. From studies in experimental animals and in man it is thought that CsA may initiate or enhance vascular injury. CsA accelerates arteriosclerosis in the spontaneously hypertensive rat and also enhances the vascular injury occurring in experimental acute serum sickness in rabbits [17, 18]. In human renal allografts the presence of glomerular capillary thrombi have been reported during CsA treatment [19]. These observations suggest that CsA causes an increase in the inflammatory component of vascular rejection, thereby leading to heavy IgM and complement deposits in the arteriolar wall [6]. Three months after conversion, these immunofluorescence staining characteristics were not found. We can therefore conclude that the influence of CsA on the arteriolar wall may at least be partially reversible. The results of arteriolar histological examination, however, did not differ significantly before and after conversion.

All biopsy specimens showed mild to moderate interstitial fibrosis with tubular atrophy [6, 9, 20]. The degree of fibrosis was reported to be strongly related to the cumulative dose of CsA during the first six months of therapy as well as to the number of acute nephrotoxic episodes [8, 9, 21]. In our study CsA levels were frequently monitored and no excessive doses prescribed. In fact, aetiology and progress of fibrosis can only be adequately assessed when sequential biopsies have been taken, starting at the day of transplantation [22]. A kidney already injured by other factors leading to fibrosis might be more susceptible to the effect of CsA, but a prospective study showed no difference in the quantity of fibrosis after 3 months of CsA or AZA therapy [22, 23]. When patients treated with CsA were converted three months after transplantation, an improvement in renal function indicated that fibrosis should not be a problem when shortterm administration of CsA is contemplated. During a one year follow up no increase in fibrosis leading to progressive renal insufficiency was found in the converted group [24]. Our results show that even longterm treatment with CsA does not lead to progressive interstitial fibrosis or vascular changes causing irreversible functional renal impairment [25].

Kidney transplants with a normal function and treated with AZA were often found to have a prominent interstitial mononuclear cell infiltrate [26]. Interstitial cell infiltrates have also been described as a prominent feature of CsA nephrotoxicity, not only in renal allografts but also in normal native kidneys in patients treated with CsA for uveitis [2, 3, 4, 13, 26, 27]. After one year of CsA therapy we found a focal infiltration primarily consisting of mononuclear cells in all but one patient [24]. The absence of eosinophils in these infiltrates merely excludes the possibility of a hypersensitive reaction as the cause of the interstitial nephritis. Classification of infiltrates as diffuse or local does not show a relation to the degree of renal impairment. When rejection episodes occurred during therapy with CsA, mostly diffuse but also focal infiltrates were reported [4, 22]. The conclusion that cellular

infiltrates in renal allografts are not specifically associated with CsA, is supported by the quantitative observation that infiltrating cells are even more numerous in grafts treated with AZA [28].

The question of whether any infiltrate in renal allograft biopsies is potentially harmful remains to be answered. It has been suggested that cell infiltrates are all attributable to varying degrees and stages of immunological reaction to allogenic tissues. A relation between cellular rejection and so called clinical rejection is not clear cut [10, 22]. In our study a substantial increase in the extent of the infiltrates was found without any clinical evidence of rejection three months after conversion. The most prominent finding was an increased incidence of invasion of mononuclear cells between tubular epithelial cells. Remarkably, infiltrating mononuclear cells invading tubular epithelium have been a hallmark of rejection for years but now seems to be compatible with a stable graft function [13]. As rejection episodes did not occur during our 18 months follow up it seems that inflammatory cells observed in stable allografts represent a very attenuated rejection process, perhaps only threatening the survival on the very long term. Alternatively, the mononuclear cells in well functioning grafts might reflect the immunological quiescence due to the development of immune regulating mechanisms involving circulating antibodies and suppressor cells. The invasion of mononuclear cells between tubular epithelium suggests that these cells are not quiescent but really represent an active cell population. Development of a florid clinical rejection episode shortly after conversion suggests that in some patients there is a rapid proliferation of cytotoxic effector cells on withdrawal of CsA which escapes the regulating mechanisms. The clinical improvement in renal function following conversion appears to be due to abolition of CsA nephrotoxicity rather than control of a low grade rejection process.

In conclusion, our study shows that histopathological features indicative of compromised renal function induced by CsA include isometric proximal tubular cell vacuolisation and arteriolar lesions. This CsA associated arteriolopathy consists of insudative lesions and IgM/complement deposits. After changing therapy to AZA, no isometric vacuolisation was found and the arteriolar deposits of IgM/complement were considerably less. There is no evidence yet that mild to moderate degrees of interstitial fibrosis cause irreversible graft dysfunction. However, conversion is accompanied by an increase in mononuclear cell infiltration, together with tubulitis. These remarkable morphological findings combined with the clinical improvement of renal function in all patients observed, is a new contribution to the discussion of the hazards of longterm treatment with CsA.

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CHAPTER 6

PHENOTYPICAL ANALYSIS OF INFILTRATES

INTRODUCTION

In chapter 5 we reported on the histology of biopsies from stable kidney allografts routinely taken after one year of continuous cyclosporin A (CsA) therapy. Typical morphological lesions, indicative of CsA nephrotoxicity, included isometric vacuolisation in proximal tubular epithelial cells and arteriolar deposits of IgM and complement factors. In an attempt to halt the adverse effect of CsA on the kidney, several transplantation groups switched the immunosuppressive regimen from CsA to azathioprine (AZA) several months after transplantation [1]. We performed such a conversion at one year after transplantation. In control renal biopsies taken three months after conversion we observed a significant regression of the CsA induced morphological lesions. At the same time, we also noticed a striking increase in the interstitial mononuclear cell infiltrates in combination with tubulitis. Although this suggested graft rejection, we did not find clinical evidence for this. On the contrary, renal function improved in all patients and remained stable during a 18 months follow-up period. In this chapter we study in more detail this phenomenon of mononuclear cells infiltrating the renal allograft in absence of rejection after conversion from CsA to AZA therapy. Monoclonal antibodies were used for phenotypical characterization and quantification of these cells.

PATIENTS AND METHODS

Fourteen recipients of a cadaveric renal allograft were studied as outlined in chapter 5. They were successfully converted from CsA to AZA one year after transplantation and renal function remained stable during a follow-up period of 18 months. Low dose prednisone was continued throughout the study. From all patients a percutaneous Tru-cut needle biopsy of the kidney allograft before and three months after conversion was obtained. Part of the biopsy specimen was fixed in 4% formaldehyde and embedded in paraffin wax. For light microscopic examination, serial 2 μm thick sections were cut from paraffin- embedded tissue and stained with haematoxylin-azafloxin, Jones and Jones Azan. Another part of the biopsy specimen was quickly frozen in liquid nitrogen and stored. This biopsy material was partly used for immunomorphological studies with monoclonal antibodies. A panel of monoclonal antisera (Becton Dickinson, U.S.A.) was applied to assess the antigenic determinants of the mononuclear cells infiltrating the graft tissue (table I).

Table I
Monoclonal antibodies used in this study.

monoclonal antibody	specificity
anti-Leu-2a	suppressor/cytotoxic T cells (CD 8+)
anti-Leu-3a	helper/inducer T cells (CD 4+)
anti-Leu-4	T lymphocytes (CD 3+)
anti-Leu-7	natural killer cells
anti-Leu-14	B cells (CD 22+)
anti-Leu-M3	monocytes/macrophages (CD 14+)

Cryostat sections were stained by a standard indirect immunoperoxidase technique. Cells with a plasma membrane distinctly stained brown were considered positive. After all biopsies were coded, light microscopic examination was performed by two observers unaware of the treatment that each patient had received at the moment of biopsy. As described in the chapter on histology, the following characteristics of the interstitium were assessed and graded according to severity on a semiquantitative scale (absent, mild, moderate and severe): cellular infiltrates classified as diffuse or focal, consisting of mononuclear or polynuclear (neutrophilic and eosinophilic) cells; presence of tubulitis (mononuclear cells infiltrating the tubular epithelium) and vasculitis (mononuclear cells infiltrating the vessel wall). After immunohistochemical labelling with monoclonal antibodies the percentage of positively staining cells was estimated semiquantitatively and expressed as a percentage [i.e. 5, 10, 20 , 30% etc.] of the total number of infiltrating mononuclear cells in at least four high power fields. Repeated scoring of the percentage of positively staining cells yielded reproducible results. The ratio of CD 4+ and CD 8+ cells was calculated by dividing the estimated percentages of Leu-3a reactive cells by that of the Leu-2a reactive cells.
Statistical analysis was performed using the Student's t test for paired data, the Mann Whitney U test and the Fisher's exact test.

RESULTS

In our studygroup of 14 patients median serum creatinine before conversion was 165 (range 103-255) μmol/l and fell to 131 (95-168) μmol/l three months after conversion ($p < 0.01$) with a concomitant increase in median creatinine clearance from 44 (range 31-72) ml/min to 59 (50-100) ml/min ($p < 0.01$).
From all 14 patients sufficient biopsy material for light microscopic examination was obtained and with all monoclonal antibodies satisfactory indirect immunoperoxidase staining was achieved. Anti-Leu-4 stained occasionally tubular brush-borders with varying intensity, but this additional staining reaction did not interfere with our scoring technique.

Before conversion, focal cellular infiltrates were present in 13/14 renal allograft biopsies. Their intensity ranged from mild to moderate. Mononuclear cells predominated over a variable but small admixture of polynuclear cells. A minimal infiltration of mononuclear cells between the epithelial tubular cells was found in only two biopsies. Phenotypical characterization of the mononuclear cells showed 56 ± 8% Leu-4+ (CD 3+) cells. The percentages of Leu-14+ (B) cells, Leu-7+ (NK) cells and Leu-M3+ (monocytic) cells were 5 ± 2, 2 ± 1, and 2 ± 1 (table II) respectively. Within the T cell population the percentage of Leu-3a+ (CD 4+) cells was 54 ± 6% and of Leu-2a+ (CD 8+) cells 46 ± 6%. The median calculated CD 4/CD 8 ratio was 1.3 (range 0.1-4.0) (table III). With the monoclonal antibody panel used 35% of the infiltrating cells could not be identified. The percentages of unstained cells were not correlated with the CsA trough levels.

Table II
Percentages of mononuclear cell subsets in kidney allograft biopsy specimens before (CsA) and three months after conversion (AZA) (mean ± SEM).

		CsA	AZA
Leu-4+	(pan-T cells)	56 ± 8	84 ± 3 **
Leu-7+	(NK cells)	2 ± 1	2 ± 1
Leu-14+	(B cells)	5 ± 2	10 ± 3
Leu-M3+	(monocytes)	2 ± 1	3 ± 1

** $p < 0.01$

Table III
Relative proportion of CD 4+ and CD 8+ cells within the T cell population (mean ± SEM), before (CsA) and three months after conversion (AZA). Ratio is expressed as median (range).

	CsA	AZA
(CD 4+)	54 ± 6	73 ± 3 *
(CD 8+)	46 ± 6	27 ± 3 *
CD 4/CD 8 ratio	1.3 (0.1 - 4.0)	2.7 (1.3 - 8.1) *

* $p < 0.05$

Three months after conversion of immunosuppressive therapy from CsA to AZA the light microscopic appearance of the interstitium showed a significant increase in the size of the cellular infiltrates ($p < 0.01$). The location was still focal in eleven but had become diffuse in three patients. These infiltrates mainly consisted of mononuclear cells. In nine biopsy specimens a marked increase of mononuclear cells between the epithelial tubular cells (tubulitis) was found ($p < 0.01$). A focal infiltration of mononuclear cells within the arteriolar wall was present in one patient. Phenotyping of the mononuclear cells within the infiltrates showed 84 ± 3% Leu-4+ (CD 3+) cells versus 56 ± 8% before conversion ($p < 0.01$, table II). The other mononuclear cell subsets tested (B cells, NK cells and monocytes) showed no significant changes after conversion (fig. 1). T cell subsets showed a rise in Leu-3a+ (CD 4+) cells from 54 ± 6% to 73 ± 3% ($p < 0.05$), while the percentage of Leu-2a+ (CD 8+) cells decreased from 46 ± 6% to 27 ± 3% ($p < 0.05$, fig. 2). The median calculated CD 4/CD 8 ratio after conversion was 2.7 (range 1.3 - 8.1) compared to 1.3 (range 0.1 - 4.0) during CsA therapy ($p < 0.05$). In all postconversion biopsies the CD 4/CD 8 ratio exceeded 1.0 (table III). No relationship could be established between morphological or phenotypical changes and improvement in kidney allograft function.

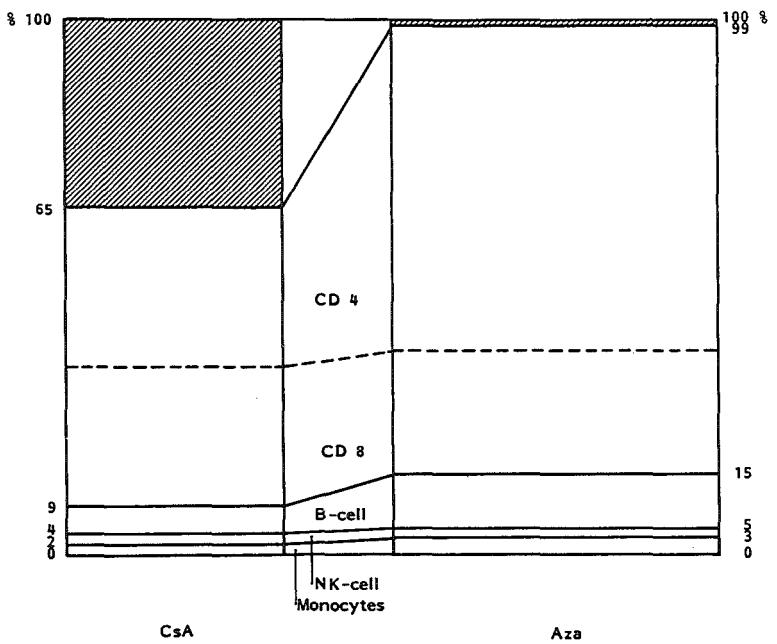

Figure 1 Mononuclear subsets in kidney allograft biopsy specimens during cyclosporin A (CsA) therapy and three months after conversion to azathioprine (AZA) therapy (mean percentages).

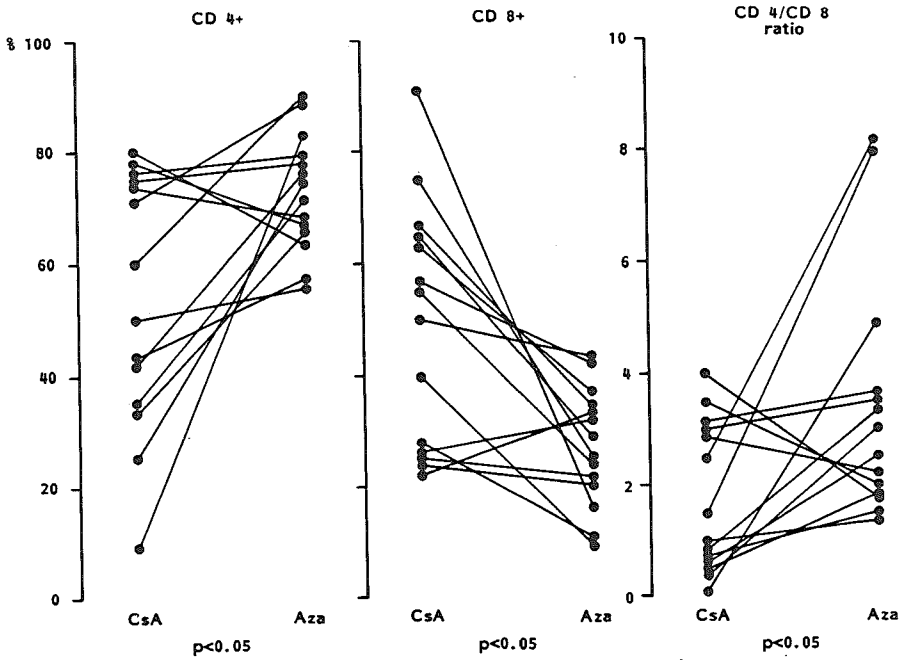

Figure 2 Percentages of CD 4+ and CD 8+ T cells and CD 4/CD 8 ratio in the individual kidney allograft biopsy before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA).

DISCUSSION

The use of monoclonal antibodies has provided new insights into the histopathology associated with graft infiltration by defining the precise subpopulations present within the often morphologically indistinct mononuclear cell infiltrates [2, 3, 4]. Various reports have dealt with the differentiation between CsA induced nephrotoxicity and rejection by phenotypical characterization of the cellular infiltrates in case of renal dysfunction [5, 6]. However, the extent and composition of the infiltrates in longterm well-functioning kidney allografts and the influence of immunosuppressive regimens on it, have not been identified.

In the present study we have tried to characterize the phenotype of the mononuclear cells invading renal allografts in the absence of rejection after conversion from CsA to AZA therapy. Before conversion we found a slight preponderance (56%) of T lymphocytes (CD 3+ cells) in small focal interstitial infiltrates. Within the T cell population, the CD 4+ cells slightly dominated the CD 8+ cells resulting

in a median CD 4/CD 8 ratio of 1.3. The contribution of B cells, NK cells and monocytes was only minor [5]. Surprisingly, 35% of the cells in the infiltrates could not be identified with the monoclonal antibodies used. The presence of neutrophilic and eosinophilic polymorphonuclear cells, or even non-inflammatory cells might be of relevance to the unstained population. However, at light microscopic examination only a very small admixture of these cells was found in the interstitial infiltrates.

A mononuclear cell population not expressing surface markers, or silent cells, has not been described before in kidney allograft biopsies. Recently, McWhinnie et al. reported on the phenotypical composition of cellular infiltrates in biopsies of CsA treated patients with stable graft function three months after transplantation [6]. Forty percent of the leukocyte infiltrate was composed of CD 3+ cells, while the other cells were mainly macrophages. An almost equal percentage of CD 4+ and CD 8+ cells was found but the presence or absence of non-staining mononuclear cells was not mentioned. In contrast, non-staining infiltrating mononuclear cells have been reported in CsA treated cardiac allografts in the absence of rejection [7]. Although this finding was not discussed in any detail, Pomerance and Stovin tried to identify the nature of these endocardial infiltrates and they suggested that these cells are of histiocytic origin [8]. However we did not find evidence for the presence of macrophages in our material using monoclonal antibodies, but the timing of biopsies after organ transplantation is obviously important and might explain the discrepancies between the various studies. We are not aware of infiltrate analyses in biopsies of stable kidney or cardiac allografts in patients treated for one year with CsA. During the process of engraftment suppression of immunological activity with loss or modulation of cell surface markers can be imagined, as has been described for MHC products in preclinical studies [9, 10]. In fine needle aspiration biopsies of kidney allografts, during quiescent periods, 50% of the infiltrating cells was found to be T cells but no mention was made of nonstaining cells [11, 12].

Three months after conversion from CsA to AZA we found a significant increase in the number of cells infiltrating the interstitium. Invasion of the tubular epithelium and even the arteriolar wall was found. Phenotypical analysis showed that the majority of these cells (84%) were CD 3+ cells. Under AZA treatment the relative proportion of the CD 4+ cells increased from 54 to 73%, while that of the CD 8+ cells decreased. The number of B cells, NK cells and monocytes was not affected by the change in immunosuppressive regimen. It should be noted that in contrast to the situation under CsA therapy, after conversion to AZA all infiltrating mononuclear cells could be phenotypically analysed.

Our finding that the majority of the cells infiltrating the graft in AZA treated renal allograft recipients with stable function are T lymphocytes, is in agreement with the findings of Burdick et al. [13]. We showed that these T lymphocytes are predominantly CD 4+ cells and both their absolute and relative numbers increase after conversion from CsA to AZA causing an increase of the CD 4/CD 8 ratio. While

the mononuclear invasion between tubular epithelial cells might represent rejection we found no clinical evidence for graft function loss. This is in line with the reports of Platt et al., Hancock et al., Waltzer et al. and Sako et al. who observed during acute rejection an increase in CD 8+ cells and not in CD 4+ cells (and consequently a fall in CD 4/CD 8 ratio) frequently accompanied by a rise of monocytes and NK cells [5, 14, 15, 16]. In contrast other investigators have reported a preponderance of CD 4+ cells during rejection [17]. These results are difficult to explain. It may be that the interpretation of staining is a source of discrepancy especially if staining is weak or poor or that the technique used influences outcome. The CD 4+ cell predominance after conversion could be due to expression of surface markers on the initially silent mononuclear cells present under CsA therapy. Withdrawal of CsA restores Interleukine-2 production capacity and the permanent antigenic stimulus of renal allograft tissue will activate resting (silent) T cells resulting in generation of more T cells, leading to an increased size of the infiltrates and invasion of the tubular epithelium. Our finding that this phenomenon did not lead to rejection, might reflect the formation of a new immunological balance between host and transplanted organ after switching the immunosuppressive regimen.

In conclusion, after successfull conversion from CsA to AZA we found a striking increase in the interstitial mononuclear cell infiltrates in the absence of rejection. Phenotypical analysis revealed that these cells were predominantly CD 4+ lymphocytes. The disappearance of cells not expressing surface makers, which are present during CsA, but not AZA, suggests that the CD 4+ cells under AZA therapy are derived from a "silent" T cell population under CsA therapy.

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CHAPTER 7

CELLULAR IMMUNE RESPONSIVENESS

INTRODUCTION

An allogeneic organ transplant represents a major immunological stimulus requiring intensive immunosuppressive therapy to prevent allograft rejection. Cyclosporin A (CsA) inhibits allograft rejection by directly interfering with the lymphocyte population. Recent studies showed that the number of T-helper (Th) cells in the peripheral blood was markedly reduced by CsA therapy [1]. In functional studies T lymphocytes were found to have an impaired lymphokine synthesis during CsA therapy, affecting both the production of Interleukine-2 (IL-2) and Interferon-γ (IFN-γ) [2].

Reduction of IL-2 synthesis prevents clonal expansion of activated T cells and generation of cytotoxic effector cells thus preventing rejection. IFN-γ transforms non-cytolytic natural killer (NK) cell precursors into a lytic state and also enhances the cytotoxic capacity of already active NK cells [3]. This NK cell activity is also augmented by IL-2 [4]. The cells responsible for NK activity in humans are identified as large granular lymphocytes [5]. These cells bear surface receptors for the Fc-portion of IgG expressed on target cells and mediate cytolysis through direct cell-contact without prior sensitization. Recently, monoclonal antibodies to specific structures on the NK cell surface have been produced, which can discriminate this cell population [6].

NK cells are thought to play a major role in host defence mechanisms against malignancies and viral infections. An impairment in NK activity assessed by killing of NK sensitive cultured target cells was found in recipients of kidney allografts after longterm azathioprine (AZA) and prednisone therapy [7]. These findings may partially explain the susceptibility of these patients to develop cancer and viral infections [8]. NK activity was found only mildly impaired under CsA treatment as compared to conventionally suppressed allograft recipients [9].

The conversion protocol enabled us to study in the same patient group under different immunosuppressive therapy regimens, the basal NK cell activity of peripheral mononuclear cells, the capacity for natural cytotoxicity by in vitro stimulation of these cells with IFN-γ and the IFN-γ production capacity after activation of peripheral blood T cells with concanavalin-A (Con-A). In addition to these functional assays, T cell and NK cell subsets were monitored by monoclonal antibodies.

PATIENTS AND METHODS

Patients

Fourteen recipients of a cadaveric renal allograft who took part in the protocol changing immunosuppressive therapy from CsA to AZA one year after transplantation, entered this study. After immunological parameters in addition to renal function studies were evaluated, CsA was stopped and the next day AZA therapy was started while continuing low dose prednisone. Three months after conversion this evaluation was repeated. Six healthy controls were included in this study for comparison of the cytotoxic assay.

Samples

A heparinized venous blood sample was drawn from each patient before and three months after stopping CsA. In addition complete white blood cell counts with differential were determined at both times. From the group of healthy volunteers only one venous sample was drawn.

NK cell assay

Lymphocyte preparation
From each patient blood samples were collected in heparinized 10 ml tubes. Peripheral blood lymphocytes were isolated by gradient centrifugation [10]. Cells were washed twice, counted and tested for viability using trypan blue dye exclusion. Viability was always more than 95%. NK cell assay was performed according to the the technique described by Tank et al. [11].

In vitro incubation of effector cells with human IFN-γ
For NK cell assay the lymphocytes were diluted in RPMI 1640 (Gibco Biocult, Scotland) containing 20% fetal calf serum (FCS) according to the chosen effector to target cell ratio 6.2 : 1, 12.5 : 1, 25 : 1 and 50 : 1, respectively. 50 µl of cell suspension was added to the wells of a round bottom microtiter plate. All assays were performed in triplicate. Human IFN-γ (Biogen, Switzerland) was diluted in RPMI 1640 to a concentration of 6.4×10^4 U/ml and 50 µl of this solution was added to the effector cells. As control wells, 50 µl of RPMI alone was added to the effector cells. The plates were incubated overnight in a humidified 5% CO_2 incubator.

Chromium (^{51}Cr) release assay for NK cell activity
Natural cytotoxicity was measured in a four hour assay using ^{51} Cr labelled K562 target cells. 2×10^6 target cells were incubated with 400 µCi of $Na_2{}^{51}CrO_4$ solution (specific activity 50 - 400 µCi/mg ^{51}Cr; Amersham, U.K.) for 1 hour at 37°C. The ^{51}Cr labelled cells were washed three times, counted and resuspended at 1×10^5/ml

in RPMI with 10% FCS. In all assays 1 x 10^4 target cells were added to the wells with control or IFN-γ treated effector cells. The microtiter plates were centrifuged for 3 minutes at 150 g and then incubated for 4 hours at 37°C. To harvest the cells, plates were centrifuged for 10 minutes at 150g and the supernatants were removed using the Skatron supernatant collection system [12]. The release of ^{51}Cr was determined by counting radioactivity in a gamma counter (LKB Wallace Ultrogamma II 1280).

Specific cytotoxicity
The percentage specific lysis in all experiments was calculated according to the formula:

$$\% \text{ specific lysis} = \frac{\text{mean experimental release} - \text{mean spontaneous release}}{\text{mean maximum release} - \text{mean spontaneous release}} \times 100$$

The maximum release was calculated by adding 10% cetavlon (ICI, U.K.) to a similar aliquot of target cells. Spontaneous release was defined as the ^{51}Cr released from target cells incubated with medium alone. This value was usually 6 - 10% of the maximum. Mean counts and standard deviations were determined in triplicate tests. The day-to-day variability of this assay was low (about 10%).

Concanavalin-A induced IFN-γ production capacity

Lymphocytes were suspended at 1 x 10^6 cells/ml RPMI 1640 containing 10% FCS, 50 IU penicillin, 50 μg streptomycin, 2 mM L-glutamine and 10^5 M 2-mercaptomethanol. One ml of this cell suspension was supplemented with 7.5 μg concanavalin-A (Con-A) (Pharmacia, Sweden) and incubated for 3 days at 37°C in a 5% CO_2 humidified incubator. The supernatant from each culture was then harvested and their antiviral activity determined in a cytopathic effect reduction assay by a dye uptake method using HEP-2 cells and 100 TCID 50 vesicular stomatitis virus as challenge [13].

Concanavalin-A stimulation assay

Lymphocyte concentration was adjusted to 7.5 x 10^6 cells/ml in RPMI 1640 medium containing 10% FCS, antibiotics, 10^{-5} M mercaptoethanol and glutamine. Cultures of 200 μl containing 2 μg Con-A were maintained for 72 hours at 37°C in a 5% CO_2 humidified incubator. Six hours prior to termination each culture was labelled with 0.8 μCi of methyl-3-H-thymidine (^{3}H-TdR, specific activity 2Ci mmol, Amersham, U.K). The cultures were harvested with an automatic harvester (microtiter, Automash, Dynatech, Holland). Cells were collected on fiberglass filters and after drying the filters were placed in scintillation vials, 3 ml scintillation fluid added and uptake of ^{3}H-TdR determined with a liquid scintillation counter (B, Searl Isocap II; efficiency 96%).

Analysis of lymphocyte subsets by monoclonal antibodies

After sedimentation of heparinized blood on a Ficoll-Hypaque gradient, subset phenotypes were quantitatively determined by immunofluorescence stains with monoclonal antibodies (Becton Dickinson Company, U.S.A.) (table I). The labelled cells were analyzed by a fluorescence activated cell sorter (FACS II, Becton Dickinson, U.S.A.) to obtain numerical percentages of each phenotype. The total lymphocyte count was determined by complete white blood cell count with differential. The actual subset counts were calculated by multiplying the percentage of each phenotype to the total lymphocyte count. The ratio of T helper (Th, CD 4+) and T suppressor-cytotoxic (Ts-c, CD 8+) cells (CD 4/CD 8 ratio) was calculated by dividing % Leu-3a+ cells by % Leu-2a+ cells.

Table I
Monoclonal antibodies used in this study.

monoclonal antibody	specificity
anti-Leu-2a	suppressor/cytotoxic T cells (CD 8+)
anti-Leu-3a	helper/inducer T cells (CD 4+)
anti-Leu-4	pan-T lymphocytes (CD 3+)
anti-Leu-7	large granular lymphocytes
anti-Leu-11a	Fc-IgG receptor on NK cell (CD 16+)
anti-Leu-16	B cells (CD 20+)
anti-Leu-M3	monocytes/macrophages (CD 14+)
anti-HLA-DR	B cells, monocytes/macrophages, activated T cells

Statistics

Statistical analysis was performed with the Student's t test for paired data and the Mann Whitney U test. For comparison with controls the Student's t test for unpaired data was used.

RESULTS

11/14 allograft recipients who entered this study were successfully converted from CsA to AZA one year after transplantation. In 3/14 patients a rejection episode occurred within 3 weeks after conversion from CsA to AZA and these patients were not included in the results comparing immunological parameters during CsA and AZA therapy. Consequently, from eleven patients full data before and three months after conversion were obtained.

Basal NK cell activity

The mean percentage (± SD) of cytotoxicity of healthy controls when tested against K562 target cells was 55.6 ± 20.6% at the lymphocyte to target ratio of 50 : 1. In allograft recipients using CsA for one year the percentage of NK cell acitivity against the K562 cellline at the same ratio was 36.4 ± 24.5% (n.s. vs controls). After conversion to AZA the mean percentage of specific target cell lysis by NK cells was decreased to 19.4 ± 15.4% ($p < 0.05$ vs CsA and $p < 0.01$ vs controls, fig. 1). Basal NK cell activity showed no relation with renal function. Rejection episodes post-conversion as occurred in three patients, were not heralded by a different percentage of cytotoxicity before conversion (15.6, 37.2 and 52.8%, respectively).

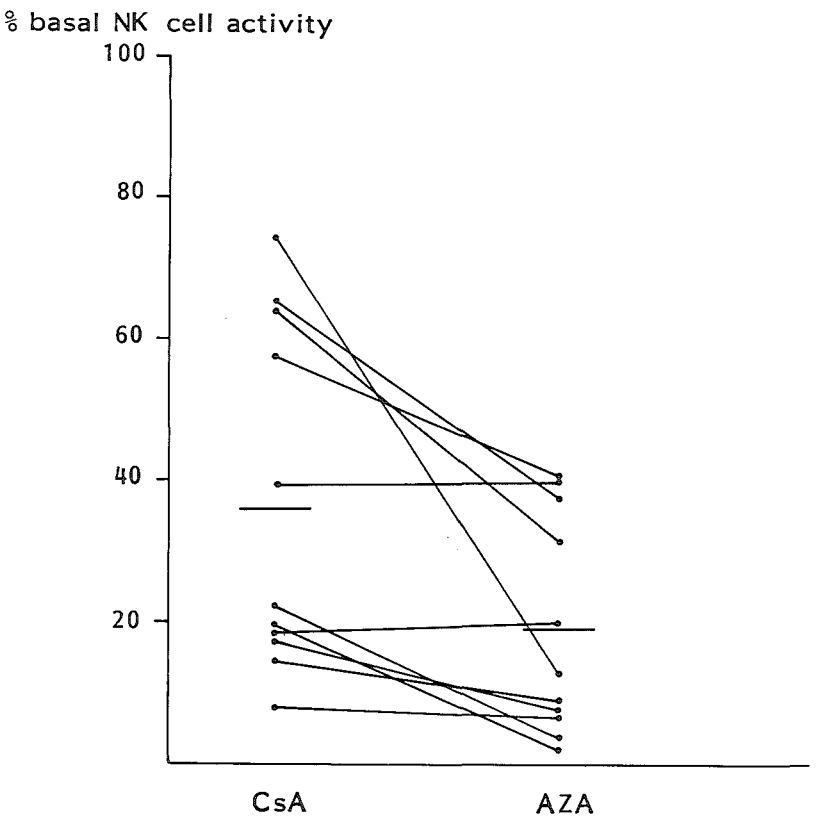

Figure 1 Basal NK cell activity against K562 cells in individual patients before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA) ($p < 0.05$).

NK cell activity after in vitro exposure to IFN-γ

Following exposure of mononuclear cells to IFN-γ, an increase of NK cell activity in the CsA treated patients from 36.4 ± 24.5% to 43.7 ± 23.0% ($p < 0.05$) was observed (fig. 2). In contrast, when mononuclear cells of AZA treated patients were exposed to IFN-γ, NK cell activity was not augmented (19.4 ± 15.4% to 22.8 ± 17.5%, n.s.). No correlation existed between basal NK reactivity and increase after IFN-γ exposure.

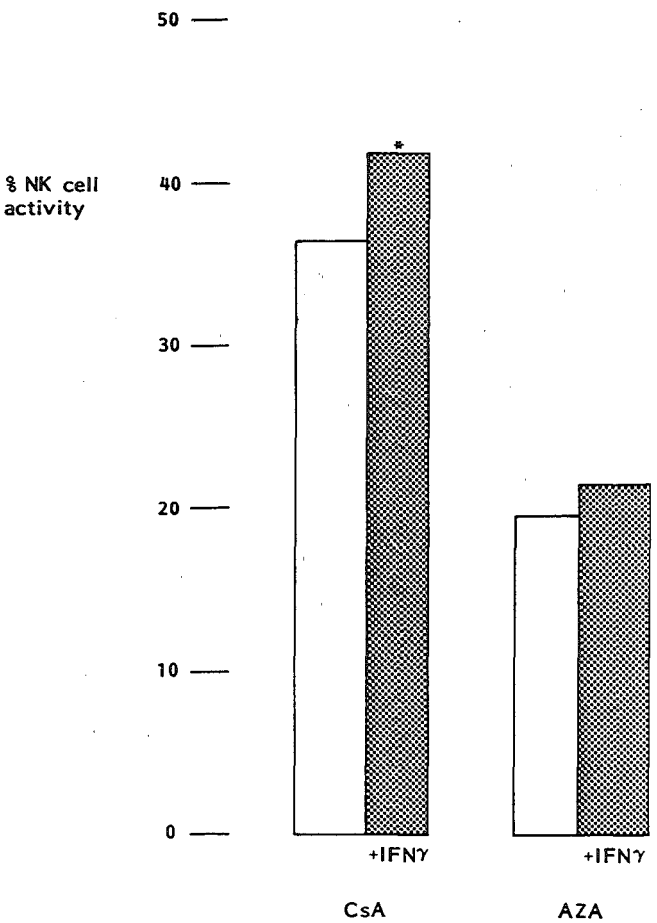

Figure 2 Mean basal and IFN-γ stimulated NK cell activity against K562 cells before and after conversion from cyclosporin A (CsA) to azathioprine (AZA) in renal allograft recipients with an effector to target ratio of 50 : 1 (* $p < 0.05$ vs. basal NK activity).

IFN-γ production capacity

After Con-A stimulation of peripheral blood mononuclear cells a median of 25 U IFN-γ per ml 10^6 cells (range < 10 - 80) was produced in CsA treated patients. After changing therapy to AZA an increase in IFN-γ production capacity was found in nine patients with a median of 80 (15-160) U IFN-γ per ml 10^6 cells. In two patients no increase in production was found, though production was not deficient with 20 and 40 U IFN-γ per ml 10^6 cells (fig.3).

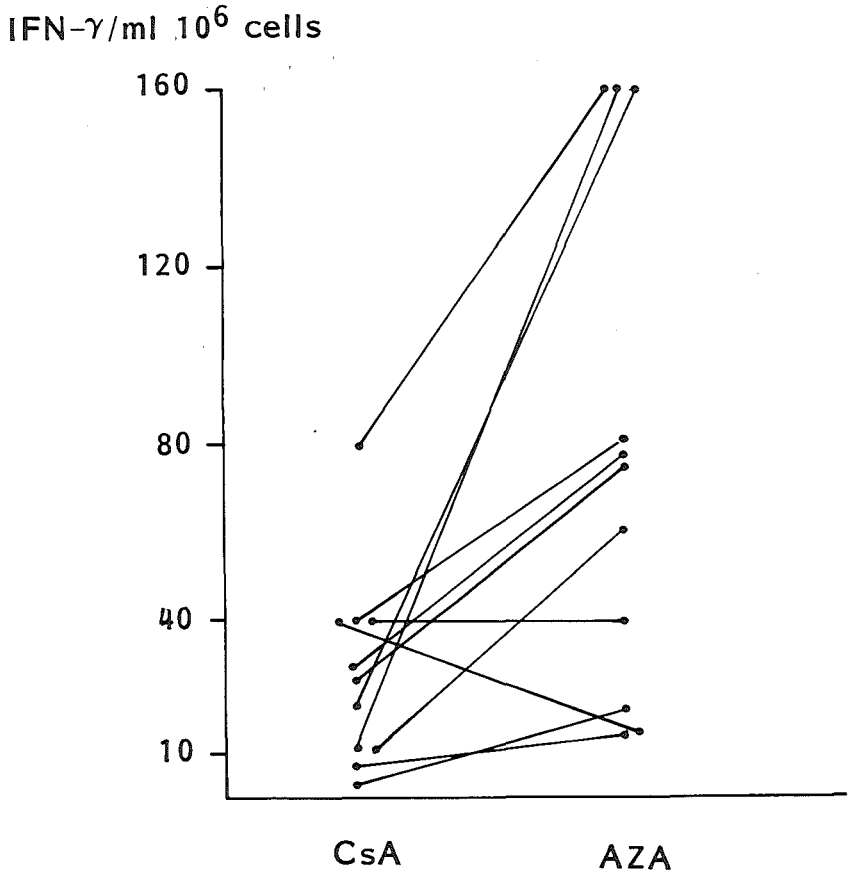

Figure 3 IFN-γ production capacity upon stimulation of peripheral blood mononuclear cells with concanavalin-A before and after conversion from cyclosporin A (CsA) to azathioprine (AZA).

Concanavalin-A stimulation of mononuclear cells

Con-A induced blastogenesis measured by ^{3}H-thymidine incorporation was not different before and after conversion (64265 ± 22871 vs 77941 ± 42671 c.p.m., n.s.).

Lymphocyte subset monitoring

In CsA treated patients the white blood cell (WBC) count was 7173 ± 2052 cells/mm^3 (mean ± SD) with 23.4 ± 8.6% lymphocytes. The mean percentage (± SD) of mononuclear cells reacting with each of the monoclonal antibodies in the samples of CsA treated patients is shown in table II.

Table II
Percentages of lymphocyte subsets before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA) (mean ± SD).

	CsA	AZA	
Leu-4+	63.3 ± 12.6	61.3 ± 17.3	
Leu-7+	14.5 ± 10.1	9.2 ± 9.1	
Leu-11a+	12.9 ± 12.3	4.9 ± 2.9	*
Leu-M3+	12.2 ± 10.2	11.3 ± 3.6	
Leu-16+	8.7 ± 9.3	7.4 ± 7.4	
HLA-DR+	20.7 ± 11.7	18.5 ± 5.2	

* $p < 0.05$

Within the T cell population the mean percentage of Leu-3a+ (CD 4+) cells was 54.1% and of Leu-2a+ (CD 8+) cells 47.4% (table III). The median calculated CD 4/CD 8 ratio was 1.2 (range 0.5 - 1.7). Changing therapy to AZA caused a significant decline in the total number of white blood cells from 7173 ± 2052 before to 4900 ± 1081/mm^3 after conversion ($p < 0.01$). Although the absolute counts of lymphocytes also declined, their percentage of WBC remained stable (23.4 ± 8.6% vs 23.3 ± 11.1%, n.s.). T cell subset evaluation revealed a significant decline in the absolute cell counts too (table IV), but the mean percentage of CD 3+ cells and CD 4+ cell did not change. The percentage of CD 8+ cells decreased significantly from 47.4 ± 9.6% before to 41.6 ± 10.5% ($p < 0.05$) after conversion. Consequently the median calculated CD 4/CD 8 ratio rose to 1.5 (range 0.8 - 2.9) ($p < 0.05$, table III). The mean percentage of mononuclear cells expressing the Leu-11a antigen decreased from 12.9 to 4.9% ($p < 0.05$). Before conversion the absolute counts of the Leu-11a+ (CD 16+) cells were 221 ± 207 and declined to 70 ± 33/mm3 three months after conversion ($p < 0.05$). When we compared the percentages of Leu-7+ before and after therapy conversion cells, no signficant change was found, while the absolute counts decreased from 340 ± 323 to 157 ± 139/mm^3.

Table III

Percentages of CD 4+ and CD 8+ cells within the T cell population before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA) (mean ± SD). Ratio is expressed as median (range).

	CsA	AZA	
CD 4+	52.9 ± 9.5	59.7 ± 9.8	
CD 8+	47.4 ± 9.6	41.6 ± 10.5	*
CD 4/CD 8 ratio	1.2 (0.5-1.7)	1.5 (0.8-2.9)	*

* $p < 0.05$

Table IV

Absolute cell counts in cells/mm^3 before and three months after conversion from cyclosporin A (CsA) to azathioprine (AZA) (mean ± SD).

	CsA	AZA	
Leu-4+	1069 ± 632	738 ± 388	*
Leu-3a+	561 ± 303	397 ± 205	*
Leu-2a+	510 ± 312	305 ± 168	**
Leu-7+	340 ± 323	157 ± 139	
Leu-11a+	221 ± 207	70 ± 33	*
Leu-M3+	181 ± 116	117 ± 53	*
Leu-16+	157 ± 203	88 ± 107	*
HLA-DR+	334 ± 214	200 ± 99	*

* $p < 0.05$
** $p < 0.01$

Leu-M3+ (CD 14+) mononuclear cells showed a significant decrease from 181 ± 116 to 117 ± 53/mm^3 ($p < 0.05$) but the relative percentages of these cells remained unchanged. After conversion the absolute but not relative number of Leu-16+ (CD 20+) cells was also lowered.

The percentage mononuclear cells expressing HLA-DR before and after conversion did not change.

The three patients who rejected their graft within three weeks after conversion to AZA showed no differences in lymphocyte subset monitoring before conversion compared to the eleven successfully converted allograft recipients (table V).

Table V
Percentages of lymphocyte and T cell subsets before conversion in 11 successfully converted patients and in 3 patients who suffered from a rejection after conversion from cyclosporine A (CsA) to azathioprine (AZA) (mean ± SD).

	no rejection	rejection
Leu-4+	63.3 ± 12.6	59.7 ± 8.5
Leu-3a+	52.9 ± 9.5	58.2 ± 10.1
Leu-2a+	47.4 ± 9.6	43.5 ± 5.7
Leu-7+	14.5 ± 10.1	14.0 ± 13.0
Leu-11a+	12.9 ± 12.3	8.3 ± 3.0
Leu-M3+	12.2 ± 10.2	7.4 ± 2.1
Leu-16+	8.7 ± 9.3	11.0 ± 9.6
HLA-DR+	20.7 ± 11.7	18.7 ± 5.9

DISCUSSION

After one year of continuous CsA therapy NK cell activity in stable cadaveric renal transplant recipients was significantly higher than three months after conversion to AZA and not statistically different from basal NK cell activity in healthy volunteers. In response to in vitro IFN-γ a significant increase in NK cell activity was observed in our CsA treated patients. No correlation between the percentage increase and basal level of NK activity in the individual patient was found.
IFN-γ was reported to augment NK activity in vitro by activating pre-NK cells and by enhancing the lytic activity of mature NK cells [3]. This action was not inhibited by CsA after IFN-γ exposure in vitro, confirming that CsA exerts its immunosuppressive effects in allograft recipients without an important interference with the magnitude of NK activity [14]. This supports the view that enhancement of NK activity in vitro by IFN-γ is not dependent on the availability of IL-2 to facilitate the development of mature NK cells from pre-NK cells, as CsA is known to interfere with IL-2 synthesis [4, 15, 16]. However, IL-2 may act in cooperation with IFN-γ to enhance the total NK capacity in vivo [17].
Three months after conversion of therapy to AZA, basal NK cell activity was significantly depressed. This agrees with earlier reports showing an inhibition of NK cell activity after several months of continuous AZA therapy [7, 9]. The loss of NK cell activity with AZA therapy can be attributed to a decrease of the number of active NK cells and IFN activable pre-NK cells [18].
Monitoring NK subsets, we found a significantly lower number of NK cells expressing the CD 16+ antigen after conversion. Anti-CD 16+ reacts with an antigen associated with the surface receptor for the Fc-portion of IgG present on NK cells [6]. In a recent report, cells with CD 16+ phenotype were found to be more cytotoxic than cells bearing the Leu-7 determinant [19, 20]. This is in

agreement with our findings that not only the number of circulating NK cells expressing the CD 16+ antigen, but also the cytotoxic action of NK cells after conversion to AZA therapy is depressed. In contrast, Leu-7 antigen is a marker of large granular lymphocytes whether they express T lineage antigens or NK lineage antigens [6]. This suggests that a significant portion of Leu-7+ cells does not have NK cell activity and no correlation between Leu-7 expression and NK cell activity can be expected [21]. The decrease in the number of NK cells is probably due to the anti-proliferative action of AZA [18, 22].

Three months after conversion to AZA, NK activity could not be enhanced by IFN-γ exposure suggesting a more pronounced effect of AZA on pre-NK cells rendering them non-responsive to IFN-γ [18]. A recent observation suggests that during AZA therapy NK cell activity in the posttransplant period is significantly higher in subjects rejecting their graft than in those with successful graft outcome [23]. In our study, we found no correlation between NK cell activity before conversion and a rejection episode after conversion as occurred in 3/14 patients. However, a period of increased and unopposed NK activity immediately after conversion could have attributed to graft rejection.

Not only the NK activity itself but also the generation of IFN-γ (the mediator of NK cell action) was investigated. In previous studies IFN-γ production capacity after in vitro mitogen stimulation of unprimed lymphocytes proved to be low during both AZA and CsA therapy when compared to healthy controls [24]. In AZA treated patients it was even more depressed. When therapy was switched in these studies from AZA to CsA, the Con-A induced IFN-γ production increased. These results were achieved one week after conversion. In contrast, our results show an increased IFN-γ production three months after switching therapy from CsA to AZA, suggesting a more profoundly inhibited but not deficient endogeneous IFN-γ production during CsA therapy. No correlation between mitogen induced blastogenesis and IFN-γ production by mononuclear cells was found. Our findings are in agreement with other studies on the effects of CsA and IFN-γ synthesis in vitro [25]. A possible explanation for the discrepancy between the results after switching therapy can be the time period between the consecutive measurements and the direction of conversion. On long term, AZA may gradually extinguish the capacity to produce IFN-γ in response to mitogen stimulation due to interference with IFN-γ synthesis or to a negative influence of inadequately functioning NK cells. The decline in NK activity occurring after introduction of AZA can represent the initiation of these events. The finding of severely decreased IFN-γ production capacity after long term AZA can also be an argument in favor of this hypothesis [24].

A near normal basal NK activity in combination with normal enhancement of specific lysis after exogeneous IFN-γ in vitro supports the view that CsA presumably does not affect NK cells themselves. However, mitogen induced synthesis of

IFN-γ, produced by T lymphocytes, is inhibited by CsA. This finding shows that not only the production of IL-2 but also of IFN-γ and possibly other lymphokines by T lymphocytes can be affected by pretreatment with CsA.

Phenotypical characterization of the circulating mononuclear cells has attracted much attention in transplantation immunology in an attempt to find the relation between immunological state and phenotype of circulating mononuclear cells [26]. T cell subset monitoring has been extensively studied but no conclusive results in predicting acute allograft rejection from the imbalance of T cell subsets have been obtained [1]. Also, in our study no predictive value to graft outcome after conversion was assessed by monitoring T cell or other mononuclear cell subsets immediately before changing therapy. In view of the time period between subset determination and the occurrence of rejection this is perhaps not surprising because the rejection episodes did not occur within a week after conversion. Ultimately serially monitoring during the first weeks postconversion will be conclusive in this regard.

Monitoring T cell subsets in stable renal transplant recipients who were successfully converted from CsA to AZA, showed an increase in CD 4/CD 8 ratio due to a significant decrease in the CD 8+ cell population. This decrease in CD 8+ cells is apparently due to the preferentially inhibitory action of AZA on suppressor cells [27]. CD 4+ cells are also sensitive to AZA treatment but the observed decline is probably only slight because their counts were already lowered by CsA. These changes in T cell counts allowed a rise in CD 4/CD 8 ratio irrespective of allograft function. The stable graft function after conversion is consistent with an undisturbed immunological balance between suppressor and cytotoxic T cells suggesting also a decline in cytotoxic cells due to AZA, provided the suppressor and cytotoxic function is not exhibited by the same cell [28].

CsA was reported to cause low counts of CD 4+ cells with preserved numbers of CD 8+ cells leading to a low CD 4/CD 8 ratio. T suppressor cells are spared from CsA inhibition while cytotoxic T cell generation is impaired [29, 30]. A relation with CsA induced inhibition of IL-2 production by CD 4+ cells for differentiation and clonal expansion of cytotoxic T cells can be postulated. It was stated that after induction of immunosuppressive therapy with CsA a shift from a high to a low CD 4/CD 8 ratio due to a decline in CD 4+ cells was correlated with an adequate immunosuppression [31]. The percentage of B cells and monocytes was not altered by conversion. Also no change occurred in the percentage of cells expressing the HLA-DR antigen on their surface.

In conclusion, changing therapy from CsA to AZA resulted in a decrease in absolute numbers of all phenotypically distinct mononuclear cells studied including NK cells. Moreover, changing therapy resulted in a decline in NK activity which could not be corrected by exogenous IFN-γ. Although after conversion to AZA an enhanced IFN-γ production capacity was found in vitro, this did not result in higher NK activity in vivo.

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CHAPTER 8

HUMORAL IMMUNE RESPONSIVENESS AFTER INFLUENZA VACCINATION UNDER CYCLOSPORIN A AND AZATHIOPRINE THERAPY

INTRODUCTION

Influenza virus infections are responsible for a high incidence of morbidity and mortality in elderly patients and those with cardiac, pulmonary and kidney diseases including renal transplant recipients [1]. Annual influenza vaccination has been recommended for these individuals.
A poor antibody response to influenza vaccination was found in patients with chronic renal diseases [2]. In patients on maintenance haemodialysis, conflicting results were obtained [3, 4, 5].
Transplant recipients on immunosuppressive therapy have an impaired immune responsiveness and protecting titers against influenza virus may not be achieved by vaccination. Several studies on this subject have been performed in patients under conventional azathioprine (AZA) immunosuppression, and both normal [3, 6, 7] and impaired antibody responses have been reported [8, 9, 10].
Cyclosporin A (CsA) has now been introduced as the main immunosuppressive agent in organ transplantation, instead of AZA. We compared the differential effects of CsA and AZA treatment on the humoral immune response to influenza vaccination in renal transplant recipients. Patients on haemodialysis and healthy volunteers served as controls.

PATIENTS AND METHODS

Study population

A total of 116 subjects entered this study: 59 renal transplant recipients with life-sustaining kidney function, 28 patients on chronic intermittent haemodialysis and 29 healthy volunteers.
A first group of 38 kidney allograft recipients was on a maintenance therapy of AZA and low dose prednisone. They received a median dosage of 1.4 (range 0.7 - 2.8) mg AZA/kg body weight and 10 (range 7.5 - 20) mg prednisone daily. Their age distribution was 26-60 (median 39.5) years with an equal sex distribution. Transplantations were performed 9-154 (median 40) months before the study. Serum creatinine concentrations were 67-600 (median 113) μmol/l.

A second group consisted of 21 kidney allograft recipients who received CsA and prednisone as immunosuppressive therapy. At the time of immunisation median daily oral dosages of 5.8 (range 2.6 - 22.9) mg CsA/kg body weight and 15 (range 7.5 - 30) mg prednisone were administered. CsA plasma trough levels were aimed at 100 - 150 ng/ml measured by a polyclonal radioimmunoassay. In this group of 14 men and 7 women with an age distribution of 19 to 65 (median 45) years, transplantations were performed 1-10 (median 6) months before immunisation. The serum creatinine concentrations at the time of immunisation ranged from 123 to 493 (median 158) μmol/l. Renal allograft function between AZA and CsA treated patients was not statistically significant. None of the kidney transplant recipients received anti-rejection therapy in the period from 6 weeks before to 6 weeks after immunisation.
For comparison a third group of 28 patients was studied; these were on chronic intermittent haemodialysis for the usual spectrum of end stage renal diseases. This group consisted of 8 men and 20 women, whose median age was 46 (range 19 - 72) years. A fourth group consisted of 29 healthy volunteers with an age distribution between 19 and 59 (median 29) years and they served as controls.

Influenza vaccine

A commercially available inactivated whole-virus vaccine (Influvac, Duphar, The Netherlands) was used in doses of 0.5 ml containing 10 μg hemagglutinin of A/Philippines/2/82 (H3N2) and A/Chile/1/83 (H1N1) virus, respectively, and 15 μg hemagglutinin of B/USSR/100/83 virus (B), as was advised by the World Health Organization for the winterseasons 1984 - 1986 [11].

Immunisation regimen

In each subject the trivalent influenza vaccine was administered by intramuscular injection in the upper arm on day 0. A booster immunisation with the same vaccine was performed on day 30. Sera were obtained on day 0, 30 and 60.

Viruses

Laboratory reference strains A/Philippines/2/82 (H3N2), A/Chile/1/83 (H1N1) and B/USSR/100/83 were kindly provided by Dr. J.J. Skehel (WHO World Influenza Center, London, England) and propagated in embryonated 10 to 12 day old chicken eggs. Because B/USSR/100/83 is a low avidity strain, resulting in suboptimal binding between virus and antibody in serological tests, infectious egg fluids of this strain were treated with ether according to Berlin et al and the watery phase was used in the serologic tests [12, 13].

Serology

Sera were seperated immediately after blood collection and clotting and kept frozen at - 20°C until titration. All sera were pretreated with Vibrio cholerae filtrate at 37°C for 16 hours and inactivated at 56°C for 1 hour. The hemagglutinin inhibition test was performed in micro-volumes as previously described, simultaneously in pre- and postvaccination sera [14]. Titers were expressed as the reciprocal of the serum dilution showing 50% hemagglutination inhibition with 4 hemagglutination units of the antigen. If no titer was observed (< 9), it was arbitrary recorded as 5 for calculations. Each hemagglutinin inhibition test was performed twice and geometric mean titers (GMT's) were calculated from the two experiments. As controls ferret antisera against the test antigens were included. A $\geq$ 4 titer rise in antibody titer following vaccination was considered to be a satisfactory response to the vaccine used. Protection against infection was associated with a titer $\geq$ 100 for influenza A and $\geq$ 200 for influenza B virus [15, 16].

Statistical analysis

Seroconversion to protecting titers and $\geq$ 4 titer rise were compared with the chi-square test. Differences in GMT's were compared with the Wilcoxon rank test.

RESULTS

Influenza vaccination induced no major side effect in any of the study groups. No evidence for immunisation associated rejection was found in the transplant recipients.

The efficacy of influenza vaccination in the four groups was studied on the basis of four indices: the GMT's reached, a $\geq$ 4 titer rise, seroconversion to protective titers and the effect of booster immunisation in patients who responded with non-protective titers to the first vaccination.

Before immunisation no significant differences in GMT's against the 3 virus strains were found among the four study groups.

The first immunisation resulted in a significant increase in GMT's in all groups to each of the vaccine components in comparison with pre-immunisation titers. CsA treated patients reached statistically significant ($p < 0.05$) lower antibody levels to the influenza A antigens (H1N1, H3N2) than both AZA treated patients and healthy controls (table I). In addition, they also showed significantly lower GMT's to influenza B than the controls. Patients on haemodialysis had significantly lower GMT's than the controls to all viruses tested. No difference was observed in antibody levels to any of the vaccine components between AZA treated patients and controls.

Table I
Mean log geometric mean titer after primovaccination with trivalent influenza vaccine in renal transplant recipients treated with azathioprine (AZA) or cyclosporin A (CsA), in patients on haemodialysis and in controls.

	controls	AZA	CsA	haemodialysis
A/Philippines/2/82 (H3N2)	2.69	2.44	2.04 *+	2.20 *
A/Chile/1/83 (H1N1)	2.49	2.56	2.06 *+	2.00 *+
B/USSR/100/83	2.70	2.40	2.16 *	2.01 *+

* $p < 0.05$ vs controls
\+ $p < 0.05$ vs AZA

The incidence of patients showing a $\geq$ 4 titer rise is shown in figure 1. We found significant differences between CsA vs AZA treated kidney allograft recipients for H3N2 (57.0% vs 81.0%), for H1N1 (52.4% vs 81.6%) and for B (31.5% vs 73.3%). CsA treated patients also did significantly less well than the control group in response to H3N2 (57.0% vs 82.8%) and influenza B (31.5% vs 70.7%). No statistical difference was seen between the healthy controls and AZA treated patients. Between patients on haemodialysis and the healthy controls a significant difference was established for influenza B (35.6% vs 70.7%) but not for the A viruses. In figure 2 the percentages of seroconversion from non-protective pre-vaccination titers to protective post-vaccination titers are shown. CsA treated patients reached protective titers to H3N2 and H1N1 in a significantly lower proportion than AZA treated patients (52.6% vs 79.4% and 55.6% vs 82.4%, respectively). For H3N2 and influenza B a significant difference in achieving protective titers was established between the control group vs CsA treated patients (85.2% vs 52.6% and 80.8% vs 23.5%, respectively). Between the control group and patients on haemodialysis a statistical difference was found for H3N2 and influenza B (85.2% vs 58.3% and 80.8% vs 32.1%, respectively). AZA treated patients showed responses that did not differ from those of the control group against both influenza A viruses while their lower conversion rate to protective titers against influenza B just reached statistical significance (47.2 vs 80.8%, $p < 0.05$) (table II).
Booster immunisation was performed in 103 of the 116 individuals investigated (table II). In subjects not responding with protective titers to the first vaccination, booster immunisation proved effective in inducing protective titers to any of the viruses in 2/14 controls, 2/23 AZA treated, 2/17 CsA treated and 6/24 haemodialysis patients (table III).

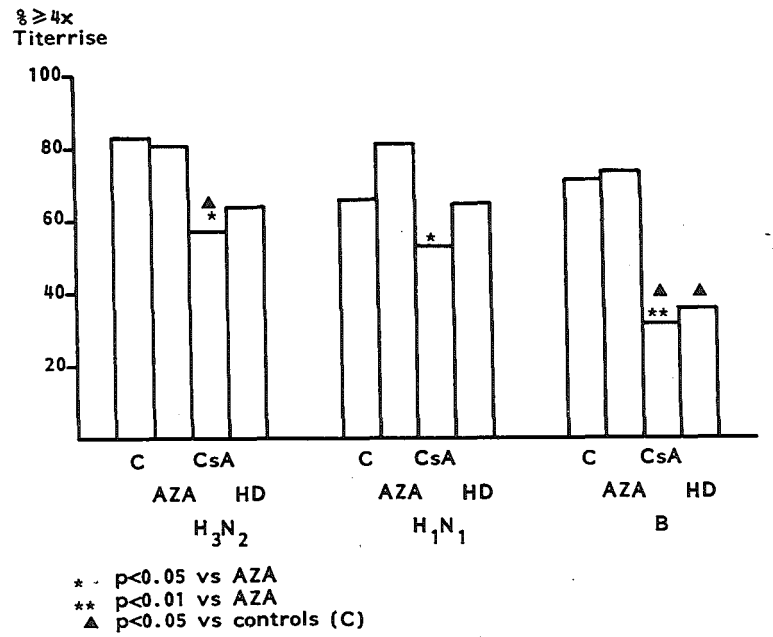

Figure 1 Fourfold or more titer rise (%) after primovaccination with trivalent influenza vaccine in controls (C), in renal transplant recipients treated with azathioprine (AZA) or cyclosporin A (CsA) and in patients on haemodialysis (HD).

Table II

Seroconversion rates to protective titers after primo- and booster vaccination with trivalent influenza vaccine in renal transplant recipients treated with azathioprine (AZA) or cyclosporin A (CsA), in patients on haemodialysis and in controls.

Seroconversion after primovaccination (%)	H3N2	H1N1	B
controls	23/27 (85.2)	19/25 (76)	21/26 (80.8)
AZA	27/34 (79.4)	28/34 (82.4)	17/36 (47.2) **
CsA	10/19 (52.6)*+	10/18 (55.6)+	4/17 (23.5) **
haemodialysis	14/24 (58.3)*	16/27 (59.3)	9/28 (32.1) **

Seroconversion after booster vaccination (%)	H3N2	H1N1	B
controls	23/27 (85.2)	20/25 (80)	23/26 (88.5)
AZA	28/31 (90.3)	28/31 (90.3)	18/32 (56.3) **
CsA	11/17 (64.7)*	10/17 (58.8)+	5/18 (27.8) **
haemodialysis	19/24 (79.2)	16/26 (61.5)	10/27 (37.0) **

* $p < 0.05$ vs controls, ** $p < 0.01$ vs controls, + $p < 0.05$ vs AZA

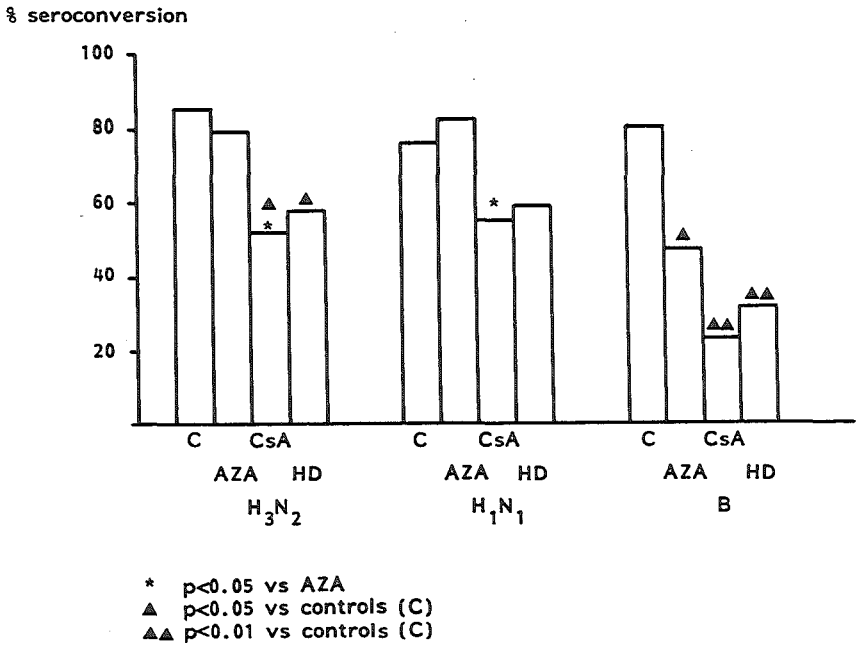

Figure 2 Seroconversion from non-protective prevaccination titers to protective postvaccination titers (%) after vaccination with trivalent influenza vaccine in controls (C), in renal transplant recipients treated with azathioprine (AZA) or cyclosporin A (CsA) and in patients on haemodialysis (HD).

Table III
Booster immunisation with trivalent influenza vaccine resulting in protective titers in patients unprotected by primovaccination.

	H3N2	H1N1	B
controls	0/4	1/6	2/5
AZA	1/4	0/3	1/15
CsA	1/7	0/7	1/11
haemodialysis	5/10	0/10	1/18

DISCUSSION

The present study shows a difference in humoral immune response following influenza vaccination between renal transplant recipients treated with CsA and those on conventional AZA therapy. Patients on CsA responded to a trivalent influenza vaccine with significant lower GMT's than patients on AZA. There were also fewer patients in the CsA group who developed a $\geq$ 4 titer rise or seroconversion to protective titers. Factors that could possibly influence the antibody response to vaccination such as age, presence of pre-vaccination antibody levels, impaired renal function or treatment with steroids were equally distributed in the CsA and AZA group. Therefore it is reasonable to assume that the difference in humoral immune responsiveness results from the different immunosuppressive agents used [17].

Antibody formation against influenza antigen is a T cell dependent phenomenon. The three principal steps leading to the production of antibodies are: specific activation of the virgin or memory B cell and proliferation and differentiation into clones of antibody producing cells. After binding of the antigen with the immunoglobulin on the B cell surface, B cell stimulating factor (Interleukin-4) is required for clonal expansion of the B cell and B cell differentiation factor is required to become a specialized immunoglobulin-secreting plasma cell [18]. Both factors are lymphokines produced by activated helper T cells. In experimental animals the humoral immune response against influenza antigens is impaired when the helper effect of T cells is lacking [19]. CsA modulates the immunoregulatory response of T cells by blocking the synthesis and release of lymphokines, thereby among other things inhibiting T cell dependent B cell activation [20, 21]. This can explain the diminished antibody response to the T cell dependent influenza antigens between CsA patients vs controls.

It has been reported before that CsA also inhibits the generation of virus-specific and cross-reactive anti-influenza cytotoxic T cell responses, that are important in host recovery from natural infection [20, 22]. So the humoral and cell-mediated immunity are suppressed in CsA treated transplant recipients.

The apparently normal humoral response to influenza vaccination of the AZA treated patients is difficult to explain because the precise mode of action of AZA is unclear. Admittedly, in vitro studies suggest that AZA suppresses the primary antibody response to T cell dependent antigens [23]. However, peripheral blood lymphocytes from AZA treated transplant recipients seem to have a normal ability to mount a primary in vitro antibody response [24]. This fits with the present observation that AZA treated patients showed a humoral immune response similar to that of the healthy control group. The normal antibody response after influenza vaccination in AZA treated patients suggests that the influence of AZA on the humoral response to T cell dependent antigens does not lead to clinical consequences. This finding is in agreement with most other studies [3, 6, 7], although there are reports of an impaired antibody response to vaccination in AZA treated renal transplant recipients with poor allograft function [8, 9].

Cellular immunity to influenza virus antigens in AZA treated patients was also reported to be normal [7].

Patients on haemodialysis showed a lower antibody response to influenza vaccination. Recently we confirmed this finding in a larger group of patients on haemodialysis after influenza vaccination against the same three viruses [25, 26]. However, in a group of patients on continuous ambulatory peritoneal dialysis (CAPD) we found a normal immune response to vaccination suggesting a intact humoral and cellular immunity in these patients. This is possibly due to a better but also a constant clearance of toxic factors by CAPD as compared to haemodialysis, preventing the development of functional lesions in the T cell system [27].

Finally we found that booster immunisation marginally improved the efficacy of vaccination against the three influenza viruses, not only in transplant recipients treated with CsA or AZA but also in patients on hemodialysis.

In summary, we have shown that CsA, but not AZA, impaired the humoral immune response after influenza vaccination in renal transplant recipients, probably due to a different mode of immunosuppressive action.

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CHAPTER 9

SUMMARY

Prevention of graft destruction by the immune system is essential in organ transplantation. In the early sixties, the combination of azathioprine (AZA) and corticosteroids proved to be a relatively effective maintenance immunosuppression in clinical kidney transplantation. The immunosuppressive protocols underwent a major change with the introduction of cyclosporin A (CsA) in 1978. Subsequently, the incidence of rejection episodes declined and one year graft survival rates significantly improved. A serious complication of CsA therapy, especially in renal transplantation, was nephrotoxicity. Because of alarming reports on progressive and irreversible renal dysfunction due to longterm CsA therapy, conversion to conventional immunosuppressive treatment after an initial inductive period was advocated. Other centers have shown that switching therapy at 3-6 months posttransplantation resulted in an improvement of renal function, but was associated with a substantial incidence of graft rejection and even graft loss. Therefore, in an attempt to minimize the longterm side effects induced by CsA but to preserve the longterm benefits on graft survival, CsA was continuously given for one year after transplantation and then conversion to AZA was performed.

In a prospective study 23 cadaveric renal allograft recipients with stable but compromised kidney function were electively converted from CsA to AZA at twelve months posttransplantation.

In the 16 recipients of a first kidney conversion was a safe procedure associated with a low incidence of rejection (6%) without graft loss. However, in the 7 recipients of a second allograft in 4 cases postconversion rejection was found while even graft loss was observed in 2 patients.

In the successfully converted patients the reversibility of CsA associated nephrotoxicity and hypertension was studied. Within three weeks after conversion serum creatinine, creatinine clearance and blood pressure showed a significant improvement and remained stable during an 18 months follow-up period. The increase in effective renal plasma flow and glomerular filtration rate after conversion suggested that CsA exerts an, at least in part, reversible renal vasoconstrictor effect. However, the increase in the glomerular filtration rate measured by the clearance of radiolabelled ^{125} I-thalamate was not as impressive as the rise in creatinine clearance. As the fraction of creatinine cleared by tubular secretion increased after stopping CsA, this finding suggested that CsA influences tubular secretion mechanisms. Practical consequences are not only an overestimation of renal dysfunction during CsA therapy but also an overestimation of the real

improvement in renal function after stopping CsA when serum creatinine and calculated creatinine clearance are used as parameters of renal function.
During CsA treatment the incidence of hypertension was high. After conversion the rapid decline in blood pressure demonstrated that the CsA induced vasoconstriction not only in the kidney but also elsewhere in the body, is to a large extent functional and reversible.
Disturbances in carbohydrate and lipid metabolism disappeared after stopping CsA. During CsA an impaired glucose tolerance was found. After conversion fasting glucose levels remained stable but the glucose levels two hours after oral carbohydrate loading were significantly lower. Switching to AZA normalized serum cholesterol and triglyceride levels. These improvements can be explained by a direct but reversible effect of CsA. An alternative explanation is that with the withdrawal of CsA also its negative effect on steroid clearance by the liver comes to an end. This in turn leads to lower steroid levels and consequently to an improvement of steroid related metabolic disturbances.
Light microscopic examination of kidney biopsies performed before and three months after conversion showed specific histologic alterations at the proximal tubular site. Before, but not after conversion, isometric vacuolisation of the proximal tubular epithelial cells was found. Arteriolar lesions consisting of insudative lesions and IgM/complement deposits were evident during CsA but significantly less prominent after conversion to AZA. Focal small sized mononuclear cell infiltrates were found in all but one preconversion biopsies. Phenotypical analysis of the infiltrating cells using monoclonal antibodies showed a slight preponderance of T cells with only small percentages of B cells, natural killer (NK) cells and monocytes. One third of the infiltrating cells remained unidentified, not expressing common surface markers ("silent cells"). After conversion we noticed an outspoken improvement in renal function in spite of a prominent increase in the infiltrates and even tubulitis. These infiltrates became markedly dominated by T cells which were mainly of the CD 4+ phenotype. Silent cells were not found anymore. Either these cells disappeared or CsA withdrawal allowed them to express their original surface markers.
Our protocol enabled us also to study the parameters of cellular immune responsiveness during CsA and after conversion to AZA. Longterm CsA did not affect the basal levels of NK cell activity and after in vitro exposure to interferon gamma (IFN-γ) a normal increase in NK cell activity could still be induced. After conversion to AZA both the number of NK cells and their activity decreased while incubation with IFN-γ did not result in the usual increase of NK cell activity anymore. IFN-γ production capacity of lymphocytes was more depressed during CsA than during AZA, suggesting a reversible inhibition of lymphokine production by CsA. Monitoring peripheral mononuclear cells showed a decrease in the absolute numbers of all phenotypically distinct cells after conversion. The prominent decrease in T cells with CD 8+ phenotype resulted in an increase of CD 4/CD 8 ratio.

The differential effects of CsA and AZA on the humoral immune responsiveness were studied in a separate study. After immunisation with inactivated influenza virus vaccine CsA treated kidney allograft recipients showed a significantly diminished antibody response to the T cell dependent influenza antigens. In contrast, the influenza vaccine induced antibody response in AZA treated patients did not differ from a group of healthy controls.
From this thesis we can conclude that CsA and AZA have a different effect on cellular and humoral immune responsiveness. CsA therapy is complicated by renal dysfunction, hypertension, glucose intolerance and elevation of serum lipid levels, but all these side effect are reversible after changing therapy to AZA. The CsA induced histologic lesions of the proximal tubular cells and arterioles also become less evident. After switching immunosuppressive therapy from CsA to AZA one year posttransplantation a new immunological balance is found, both in the peripheral blood and in the kidney allograft. Conversion at one year after transplantation is a safe procedure for first graft recipients preserving the benefits of longterm survival by CsA but it is contra-indicated for recipients of a second kidney allograft.

SAMENVATTING

De klinische toepassing van cyclosporine A en van azathioprine na niertransplantatie

Niertransplantatie is een geaccepteerde vorm van nierfunctie vervangende therapie bij patiënten met een terminale nierinsufficiëntie. Na transplantatie is het voorkómen van afstotingsreacties die ontstaan als donor en ontvanger niet genetisch identiek zijn, van essentieel belang. Sedert het begin van de zestiger jaren werd de combinatie van azathioprine (AZA) en corticosteroïden als immunosuppressieve onderhoudsbehandeling met redelijk succes toegepast. Het beschikbaar komen van cyclosporine A (CsA) in 1978 leidde tot een belangrijke verbetering van de resultaten na niertransplantatie. De incidentie van afstotingsreacties daalde en de éénjaars transplantaatoverleving steeg. Nefrotoxiciteit en hypertensie vormden echter ernstige complicaties van CsA behandeling, met name bij niertransplantatie patiënten. Verontrustende berichten in de literatuur voorspelden een progressief en irreversibel nierfunctieverlies ten gevolge van langdurig CsA gebruik. Aangeraden werd CsA slechts gedurende korte tijd na transplantatie te gebruiken om een goede immunologische acceptatie van het transplantaat te bewerkstelligen en vervolgens de onderhoudsbehandeling voort te zetten met de conventionele immunosuppressiva. Omzetting van immunosuppressieve therapie van CsA naar AZA, 3 tot 6 maanden na transplantatie, resulteerde weliswaar in een verbetering van de nierfunctie maar ging tevens gepaard met een verhoogde kans op afstotingsreacties en op verlies van transplantaatfunctie. Door het uitstellen van de omzetting van CsA naar AZA behandeling tot 12 maanden na niertransplantatie werd getracht een betere immunologische acceptatie van het transplantaat te bewerkstelligen, terwijl de bijwerkingen van langdurig CsA gebruik beperkt konden blijven.

In een prospectieve studie werd de immunosuppressieve therapie 12 maanden na niertransplantatie electief omgezet van CsA naar AZA bij 23 ontvangers van een postmortaal niertransplantaat met een stabiele maar gecompromitteerde nierfunctie. Bij de 16 ontvangers van een eerste transplantaat bleek conversie een veilige procedure die slechts bij één patiënt gepaard ging met een afstotingsreactie zonder transplantaatverlies. Bij 4 van de 7 ontvangers van een tweede transplantaat trad echter een afstotingsreactie op, waarbij in 2 patiënten het transplantaat zelfs verloren ging. In de groep patiënten waarbij de conversie zonder complicaties verliep werd de reversibiliteit van de aan CsA gerelateerde nefrotoxiciteit en hypertensie bestudeerd.

Binnen 3 weken na conversie toonden het serum creatinine, de creatinine klaring en de bloeddruk een significante verbetering die gedurende de vervolgperiode van

18 maanden gehandhaafd bleef. De duidelijke toename van effectieve nierdoorbloeding en glomerulaire filtratiesnelheid na conversie geeft aan dat CsA een, in ieder geval ten dele, reversibel vasoconstrictief effect op de niervaten uitoefent. De stijging van de glomerulaire filtratiesnelheid gemeten als de klaring van radioactief jodium-thalamaat was echter niet zo groot als de toename van de endogene creatinine klaring. Deze bevinding suggereert dat de hoeveelheid creatinine die wordt uitgescheiden via de tubulus, toeneemt na het stoppen van CsA. Het praktisch belang van deze bevinding is dat niet alleen de ernst van de nierfunctie stoornis tijdens CsA therapie maar ook de werkelijke verbetering van de nierfunctie na het stoppen van CsA wordt overschat, indien serum creatinine en creatinine klaring worden gebruikt als parameters van de nierfunctie.
Hypertensie is een andere veel voorkomende complicatie tijdens CsA therapie. De snelle daling van de bloeddruk na conversie toonde aan dat de door CsA veroorzaakte vasoconstrictie niet alleen in de nier maar mogelijk ook elders in het lichaam voor een belangrijk deel functioneel en reversibel is.
Na het stoppen van CsA verdwenen ook de negatieve effecten op het koolhydraat en lipiden metabolisme. Tijdens CsA therapie bestond er een gestoorde glucosetolerantie. Na conversie trad er geen verandering op van de nuchtere bloedsuikerwaarden, maar de twee uurs bloedsuikerspiegels tijdens de orale glucose tolerantie test daalden significant. Ook daalden de nuchtere cholesterol en triglyceride waarden tot binnen de normale grenzen. Deze verbeteringen kunnen worden verklaard door een direct maar reversibel effect van CsA op koolhydraat en lipiden huishouding. Een alternatieve verklaring is echter dat na conversie het remmende effect van CsA op de klaring van steroiden via de lever wegvalt en de door steroiden veroorzaakte metabole bijwerkingen verminderen.
Histologisch onderzoek van nierbiopten genomen voor en 3 maanden na conversie liet specifieke veranderingen zien in de proximale tubulus. Vóór conversie bestond er een isometrische vacuolisatie in de epitheliale cellen van de proximale tubulus. Na conversie werd dit niet teruggevonden. Afwijkingen in de arteriolen die bestonden uit insudatieve lesies en neerslagen van IgM/complement kwamen bij vrijwel alle patiënten voor tijdens CsA therapie. Na conversie naar AZA waren dergelijke lesies nog slechts aantoonbaar in een minderheid van de patiënten. Fenotypische analyse van de cellen in de interstitiele infiltraten met behulp van monoklonale antilichamen liet een geringe meerderheid van T cellen zien. B cellen, NK (natural killer) cellen en monocyten kwamen slechts in een laag percentage voor, terwijl ongeveer 1/3 van de cellen met de toegepaste monoklonale antilichamen niet kon worden geïdentificeerd. Na conversie werd ondanks een opvallende toename van infiltraat en zelfs aantasting van tubuli een verbetering in nierfunctie gevonden. De infiltraten waren voornamelijk opgebouwd uit T cellen waarbij het CD 4+ fenotype overheerste. In tegenstelling tot de situatie voor conversie konden nu alle infiltraatcellen worden getypeerd. Dit kan worden verklaard door het verdwijnen van de niet geïdentificeerde cellen na conversie of door het wegvallen van CsA afhankelijke remming van antigeen-expressie.

Ook de parameters van cellulaire immunoreactiviteit tijdens CsA en na conversie naar AZA therapie werden bestudeerd. Langdurige CsA therapie had geen invloed op de basale NK activiteit. Bij in vitro blootstelling aan interferon gamma (IFN-γ) werd een normale toename gezien in NK activiteit. Na conversie was niet alleen het aantal NK cellen maar ook de basale NK activiteit afgenomen terwijl na incubatie met IFN-γ geen toename in NK activiteit meer werd gevonden. De IFN-γ productiecapaciteit was echter tijdens CsA lager dan tijdens AZA therapie. Dit pleit voor een reversibele remming van deze lymfokine productie door CsA. Bepaling van de mononucleaire cellen in het perifere bloed liet een daling van alle fenotypisch te onderscheiden cellen zien na conversie. De op de voorgrond staande daling van T cellen met CD 8+ fenotype leidde tot een stijging in de CD 4/CD 8 ratio.

De invloed van CsA en AZA op de humorale immunoreactiviteit werd bestudeerd in een aparte studie opzet. Bij AZA behandelde niertransplantatie patiënten werd een stijging van antilichaamtiters na vaccinatie met geinactiveerd influenza virus vaccin vastgesteld die niet verschilde van een groep gezonde controle personen. Na vaccinatie werd bij CsA behandelde niertransplantatie patiënten een duidelijk verlaagde antilichaamrespons op de T cel afhankelijke influenza antigenen gevonden.

Uit de in dit proefschrift beschreven bevindingen komt naar voren dat CsA en AZA een verschillend effect op de cellulaire en humorale immunoreactiviteit hebben. De omzetting van immunosuppressieve therapie van CsA naar AZA één jaar na niertransplantatie leidt tot het ontstaan van een nieuwe immunologische balans, niet alleen in het perifere bloed maar ook in het niertransplantaat. De toepassing van CsA wordt gecompliceerd door het optreden van nierfunctievermindering, hypertensie, glucose intolerantie en stijging van serum lipiden spiegels. Ieder van deze bijwerkingen blijkt echter omkeerbaar te zijn nadat CsA is vervangen door AZA. Ook de histologische afwijkingen van de proximale tubulus cellen en van arteriolen nemen af na conversie. Omzetting van CsA naar AZA, een jaar na niertransplantatie, is een veilige procedure voor ontvangers van een eerste transplantaat waarbij de voordelen van CsA op de transplantaat overleving worden behouden. Bij ontvangers van een tweede transplantaat is deze procedure echter gecontraïndiceerd.

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Hulp en steun bij de totstandkoming van een proefschrift is onontbeerlijk.
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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 2 juni 1955 te 's-Gravenhage. De middelbare school periode aan het Eerste Vrijzinnig Christelijk Lyceum te 's-Gravenhage werd afgesloten met het behalen van het Gymnasium-β diploma in 1973. In ditzelfde jaar werd met de studie Geneeskunde aangevangen aan de Erasmus Universiteit te Rotterdam. Het artsexamen werd afgelegd in november 1979. Aansluitend trad hij in dienst van het Ministerie van Defensie als beroeps-militair-arts bij de Koninklijke Landmacht. In de periode 1980 - 1982 was hij Commandant Geneeskundige Verzorgingsgroep te 's-Gravenhage. In 1982 werd begonnen met de opleiding tot internist op de afdeling Interne Geneeskunde I, Academisch Ziekenhuis Rotterdam-Dijkzigt te Rotterdam, opleider Prof dr J Gerbrandy en later Prof dr MADH Schalekamp. Vanaf zijn inschrijving in het Specialisten Register op 1 april 1987 is hij werkzaam als internist in het Militair Hospitaal Dr A Mathijsen te Utrecht in de rang van majoor-arts.